The Effect of Transcription Factor Zhangfei/CREBZF on Osteosarcoma Cells and the Mechanisms Responsible

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ABSTRACT

Osteosarcoma (OS) is the most common primary malignant bone tumour in humans and dogs. Although medicine has made dramatic progress in treating osteosarcoma by surgery, with chemotherapy given before and after surgery, drug resistance and highly metastatic spread are often responsible for the failure of current therapies. Thus, more effective therapeutic approaches for treating osteosarcoma are needed. Previous results from our laboratory and others had shown that the basic-leucine zipper (bLZip) containing transcription factor, Zhangfei/CREBZF is a potent inhibitor of a variety of other transcription factors and has a dramatic effect on the growth of several cancer cell lines, including dog OS and human medulloblastoma cells. The objective of the studies described in this thesis was to determine the molecular mechanisms by which Zhangfei exerts its effect on dog and human OS cells.

Several stressors in the microenvironment of cancer cells directly or indirectly perturb the endoplasmic reticulum (ER), which then activates the Unfolded Protein Response (UPR). The UPR modulates the effects of stress and allows tumours to survive, develop, metastasize and escape therapy. The UPR is regulated by three bLZip transcription factors—ATF6, ATF4 and Xbp1s. Since Zhangfei inhibits Luman/CREB3, a bLZip structurally similar to and closely related to ATF6 and ATF4, I initially focused my efforts on this pathway. I hypothesized that *Zhangfei interacts with UPR-related bLZip transcription factors and inhibits their ability to activate the UPR signaling pathways, thereby suppressing the growth of cancer cells and increasing their susceptibility to ER stressors*.

To test this hypothesis, we monitored cell growth as well as levels of UPR gene transcripts and proteins in several dog and human osteosarcoma cell lines infected with adenovirus vectors expressing Zhangfei, and studied the interactions between Zhangfei and the UPR-mediator, Xbp1s. The results showed that the ectopic expression of Zhangfei in cell lines derived from dog osteosarcomas potently suppressed cell growth and inhibited their ability to activate the UPR. Further studies demonstrated that Zhangfei inhibited the UPR, at least partially, by binding to Xbp1s and suppressing its ability to

activate transcription from a promoter containing unfolded protein response elements (UPRE). The leucine zipper of Zhangfei was required for this interaction, which led to the subsequent proteasomal degradation of Xbp1s. However, we also found that the effects of Zhangfei were not universal. While Zhangfei had a profound effect on the growth and UPR in some OS cell lines, it either had only a partial effect, or no effect on others. This suggested that susceptibility (or resistance) to Zhangfei may be an inherent property of OS cell lines.

Since the suppressive effects of Zhangfei were not universal, and it had no obvious effects on untransformed cells and some cancer cell lines, I proposed that *Zhangfei mediates its effect on cell growth and the UPR through an intermediary that is either not induced or is defective in cells that are unaffected by Zhangfei*. I found that this intermediary was the tumour suppressor protein p53. The inhibitory effects of Zhangfei were only observed in the wild-type p53 expressing OS cell line U2OS while Zhangfei had no effect on the p53-null OS cell line MG63. In cells with functional p53, the ectopic expression of Zhangfei caused it to displace the ubiquitin ligase mdm2 and stabilize p53. Suppression of p53 by siRNA partially inhibited the effects of Zhangfei on the UPR and cell growth. In contrast, OS cells lacking functional p53 could be made to respond to Zhangfei if they were transfected to express wild-type p53. These results explain why Zhangfei has a profound effect on some cancer cells while having no obvious effect on others. I also characterized the interaction of Zhangfei and p53 by mapping the interacting domains on both proteins, showing that the bLZip domain of Zhangfei and the N-terminal transactivation domain (NTD) of p53 were required for their interactions.

My findings reveal the profoundly inhibitory effects of Zhangfei on OS growth and the UPR, a stress-response known to promote tumour survival. I also show how Zhangfei may exert its effects. My work suggests an alternative modality for the therapy of certain types of OS, and perhaps other tumours with functional p53.

Key words: Zhangfei/CREBZF, osteosarcoma, cell growth, UPR, p53

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List of Abbreviations

- AdLZ/Adeno-LacZ – adenovirus vector expressing beta-galactosidase
- AdZF/Adeno-ZF – adenovirus vector expressing Zhangfei
- ANOVA – analysis of variance
- ATF4 – activation transcription factor 4
- ATF6 – activation transcription factor 6
- β-gal – beta-Galactosidase
- bLZip – basic region, leucine zipper domain
- CAT – Chloramphenicol-acetyl-transferase
- CBP – Cyclic AMP responsive element binding protein-binding protein
- CEBPB – CCAAT enhancer binding protein-beta,
- CHOP – CAAT enhancer-binding protein homology protein
- CREBH – Cyclic AMP responsive element binding protein, hepatocyte specific
- CREBZF – Cyclic AMP response element binding protein, Zhangfei
- CREB3 – Cyclic AMP response element binding protein 3, also known as Luman (see below)
- CTp53 – Carboxyl terminus of 53,000 molecular weight tumour suppressor protein
- DDIT3 – DNA damage inducible transcript -3
- DNAJB9 – homologue of DNAJ/ 40 kD heat shock protein
- EDEM – ER degradation enhancer mannosidase alpha-like 1
- EDTA – ethylenediaminetetraacetic acid
- eIF2α – eukaryotic translation initiation factor 2alpha
- ER – endoplasmic reticulum
- ERAD – ER-associated protein degradation
- ERN1 – ER to nucleus signaling
- FBS – foetal bovine serum
- GAPDH – glyceraldehyde 6 phosphate dehydrogenase
- GADD34 – Growth arrest and DNA damage-inducible protein

- GRP78/Bip – glucose regulated protein 78,000 MW
- HBX – hepatitis B virus X protein
- HCF – host cell factor
- HERP – homocysteine-induced endoplasmic reticulum protein
- HERPUD1 – homocysteine-inducible ER stress inducible ubiquitin-like domain member 1
- HIFs – hypoxia inducible family of transcription factors
- HSPA1B – heat shock protein A1B, also called HSP70 kD protein 1B
- HSV-1 – Herpes simplex virus type 1
- K-bLZip – Kaposi sarcoma herpes virus bLZip protein
- ING2 – inhibitor of growth protein 2
- INSIG1 – insulin-induced gene 1
- IRE1 – inositol requiring enzyme
- IRF1 – host interferon response factor 1
- L – leucine
- LacZ – *Escherichia coli* protein beta-galactosidase
- Luman/CREB3 – Cyclic AMP response element binding protein – Luman
- MafB – host transcription factor maculoaponeurotic fibroma homologue B
- MAPK – mitogen-activated protein kinase
- mdm2 – mouse double minute homologue 2
- MEM – Eagles minimal essential medium
- MI – mock-infected
- mTOR – the kinase mammalian target of rapamycin
- NOTCH1 – Drosophila *Notch* family protein 1
- NTD – N-terminal transactivation domain of p53
- OS – osteosarcoma
- p53 – protein 53,000 molecular weight
- p53C – DNA-binding core domain of p53
- PCR – polymerase chain reaction
- PERK – putative receptor protein kinase

- PIG3 – p53-inducible protein 3
- PRR – proline-rich region of p53
- SDS – sodium dodecyl sulfate
- siRNA – small interfering RNA
- SMAD – small body size – mothers against decapentaplegic
- SMILE – small heterodimer partner interacting leucine zipper protein, also known as Zhangfei or CREBZF
- TAD – transactivation domain of p53
- TET – tetramerization domain of p53
- UPR – Unfolded Protein Response
- UPRE – Unfolded Protein Response Element
- VP16 – virion protein #16
- Xbp1 – X-factor binding protein 1
- Xbp1s – Xbp1 derived from cytoplasmic spliced mRNA

1. Introduction

1.1 Osteosarcoma (OS)

1.1.1 Canine and human OS

Osteosarcoma (OS) is the most frequent primary malignant bone tumour in children and adolescents, and its incidence in dogs is ten times greater than in humans. The high incidence of spontaneous canine OS makes the dog an attractive model candidate to study in relation to their human counterparts in a large group. In addition, dog OS bears many similarities with the OS in humans, which are difficult to replicate in other models. For instance: a) Dogs share a common environment with humans and are exposed to similar environmental contributors to tumorigenesis. b) Canine and human OS have similar microenvironments (Rankin et al. 2012). c) Compared to the immunodeficient rodents, the role of an intact immune system in the initiation and development of OS, as well as its contribution to therapeutic strategies, can be investigated in dogs. d) Similar biological and clinical features such as, male sex predilection, large patient size, appendicular site, metaphyseal location, generally unknown etiology, high-grade histology, high local aggressiveness, high genomic instability, rapid metastasizing potential, lung metastasis, and similar response to conventional therapies (reviewed by (Withrow et al. 1991; Ambron and Walters 1996; Mueller et al. 2007; Paoloni and Khanna 2008; Khanna et al. 2009). Thus, spontaneous dog osteosarcomas are excellent models for studying signaling pathways that regulate human tumour growth and for the development of therapeutic agents.

1.1.2 Current therapeutic approaches of OS

Current therapeutic approaches for OS consist of surgical resection, multi-agent chemotherapy and radiotherapy. Although long-term survival in localized OS has been dramatically improved in both humans and dogs by conventional treatments, recurrence and metastatic spread of OS always leads to a poor patient prognosis (Ando et al. 2013). For example, over 80% of patients with localized OS developed lung metastases after

amputation alone, and the mean survival time after recurrence is less than 12 months (Marina et al. 2004). Thus, innovative drugs and alternative approaches are needed to further improve outcomes for OS patients. At present, several new therapeutic agents being studied target the signal transduction pathways that are activated, inhibited or mutated in OS. These relevant signal pathways include nonreceptor tyrosine kinase Src, Mammalian Target of Rapamycin (mTOR), Hedgehog (Hh) signaling pathway, tyrosine kinase receptors (e.g receptors for vascular endothelial growth factor [VEGF], platelet-derived growth factor [PDGF], insulin-like growth factor [IGF], human epidermal growth factor receptor-2 [HER2] and hepatocyte growth factor receptor [HGFR]), as well as tumour suppressor p53 (reviewed by (Ambron and Walters 1996; Ando et al. 2013; Gill et al. 2013). In addition to these intracellular signal pathways, immunomodulators and signaling of bone metabolism are also considered as the alternative targets of the novel therapeutic agents.

1.1.3 Genetic alternation in OS

Nearly 70% of osteosarcoma tumours have genetic and molecular alternations, including abnormalities of chromosomal regions, mutation of tumour suppression genes, activation of oncogenes, as well as deregulation of signaling pathways (Tang et al. 2008). The most frequently altered genes and signaling pathways in both dogs and humans include: p53, retinoblastoma protein (Rb), Phosphatase and tensin homolog (PTEN), phosphoinositide 3-kinase/AKT (PI3K/AKT) pathways, and Mitogen-activated protein kinases (MAPK) cascade (reviewed by (Mueller et al. 2007). Many studies consider OS as a differentiation disease, because the genetic and epigenetic changes (e.g. activation of oncogenes or inactivation of p53 and RB tumour suppressor genes) disrupt osteoblast differentiation from mesenchymal stem cells and may contribute to OS development (Haydon et al. 2007). One of the most frequently detected genetic alternations involved in osteogenic differentiation and bone tumorigenesis is tumour suppressor protein p53. The association of p53 mutations with significant numbers of OS suggests that a lack of functional p53 increases susceptibility of OS. (Andreassen et al. 1993; Bodey et al. 1997; Varley 2003). Therefore, the mutation status of p53 could serve as a valuable indicator for early diagnosis, prognosis, and for predicting chemoresistance of OS (Goto et al. 1998).

1.2 Zhangfei/CREBZF

1.2.1 Overview

Zhangfei, also known as CREBZF or SMILE (Small Heterodimer Partner [SHP]-interacting leucine zipper protein), is a neuronal cellular transcription factor identified in our laboratory through its interaction with Host Cell Factor 1 (HCF-1) (Lu and Misra 2000b), a cellular protein required for the initiation of the herpes simplex virus type 1 (HSV-1) replicative cycle. Zhangfei belongs to the cyclic AMP response element-binding (CREB) protein family. Zhangfei is believed to play a role in the initiation of HSV-1 latency by inhibiting viral replication in unstressed neurons (Varley 2003; Akhova et al. 2005; Misra et al. 2005). While Zhangfei is present in mature neurons, it is not detected in developing neurons or in neuronal tumours.

1.2.2 Structure and function

Zhangfei, an important member of the basic domain-leucine zipper (bLZip) family of transcription factors, possess a basic region and a hydrophobic leucine zipper region containing multiple leucine residues at approximately 7-residue intervals. The bLZip motif of Zhangfei exhibits sequence homology with other members of the bLZip family, and like them, may form homo- and hetero-dimeric complexes through pairing of bLZip motifs, creating a DNA contact surface capable of binding to diverse cis-acting regulatory elements in gene promoters (Hogan et al. 2006). Zhangfei may interact with other transcription regulators through an acidic activation domain, an HCF-1-binding motif (HBM) and nuclear receptor binding motifs (Lu and Misra 2000b) (Fig 1.1).

Zhangfei differs from other bLZip proteins in that while it can homo- or hetero-dimerize through its leucine zipper, it is incapable of binding any consensus bLZip response elements as a homodimer. This is likely due to the absence of a critical asparagine residue in the basic region, which in other bLZip transcription factors is considered crucial to their ability to recognize response elements in gene promoters (Cockram et al. 2006). Zhangfei can, however, bind to and regulate some other bLZip transcription factors as a heterodimer with other proteins such as Luman/CREB3 (Misra et al. 2005), SMAD 1,5,8

(Lee et al. 2012a), CREBH (Misra et al. 2012), and ATF4 (Hogan et al. 2006). Zhangfei has been reported to regulate the activity of the HSV co-activator VP16 through HBM (Lu and Misra 2000b) and to act as a transcriptional co-repressor of nuclear receptors glucocorticoid receptor, constitutive androstane receptor, and hepatocyte nuclear factor 4α together with SHP (Xie et al. 2008; Xie et al. 2009a; Xie et al. 2009b). In addition, curcumin, a natural polyphenolic compound, can induce the expression of the Zhangfei gene through the activation of AMPK (Misra et al. 2012). Treatment of INS-1 rat insulinoma cells with high concentrations of glucose and palmitate (Lee et al. 2012b) as well as treatment of canine MDCK cells with amino acid deprivation (Zhang et al. 2010) can also increase Zhangfei expression (Fig 1.1). Based on the previous studies of our group, Zhangfei was implicated in cell cycle arrest and apoptosis of cancer cells through the observation that ectopic expression of Zhangfei in medulloblastoma (ONS76, UW228) and osteosarcoma (D-17) cells caused them to cease growth and eventually die (Valderrama et al. 2009; Bergeron et al. 2013).

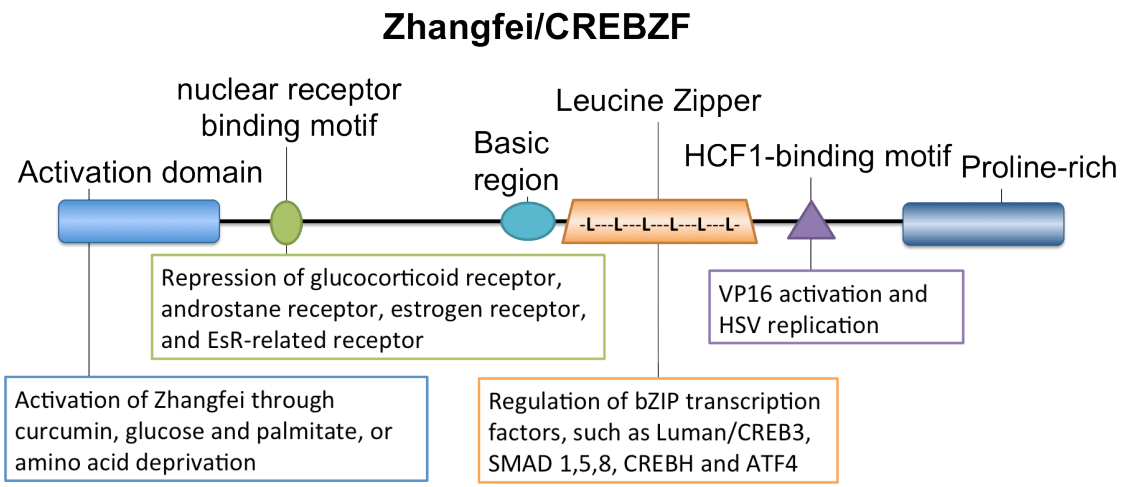

Figure 1.1 Structure, domains and functions of Zhangfei protein. The Zhangfei protein consists of an activation domain, a nuclear receptor binding motif, a basic region-leucine zipper domain (bLZip), an HCF1-binding motif (HBM), and a proline-rich region. The functions of each domain are described in 1.2.2.

1.3 Unfolded Protein Response (UPR)

1.3.1 Endoplasmic reticulum (ER) stress

The endoplasmic reticulum (ER) is an essential eukaryotic organelle where secretary and membrane proteins are synthesized and undergo a series of post-translational modifications, notably glycosylation and the formation of disulfide bonds. The ER is also responsible for the storage and regulation of calcium, the metabolism of steroids, and for the detoxification of some lipid-soluble drugs and various harmful compounds produced by metabolism in liver cells (Zhao and Ackerman 2006; Anelli and Sitia 2008).

In eukaryotic cells, newly synthesized proteins are transported into the lumen of the ER as unfolded polypeptide chains, and are folded by ER-resident chaperones. If the amount of nascent or unfolded proteins exceeds the capacity of the ER for protein maturation, the normal physiological state of the organelle is perturbed, creating ER stress (Ron and Walter 2007).

ER stress can also be induced by hypoxia, glucose deprivation, altered calcium regulation, alterations in the function of important membrane and secretory proteins, viral infection, obesity, and protein-inclusion-body diseases (Kim et al. 2008). Under these ER stress conditions, unfolded or misfolded proteins accumulate and activate adaptive intracellular stress responses that aim to alleviate ER stress and allow the cell to adapt to the new conditions (Malhotra and Kaufman 2007). One of the most important stress responses is the unfolded protein response (UPR).

1.3.2 UPR signaling pathways

The UPR is an adaptive cellular stress response that strives to alleviate ER stress and maintain ER homeostasis, and it is conserved between all mammalian species, as well as yeast and some worm organisms, such as *Caenorhabditis elegans* (Uccelletti et al. 2008; Natarajan et al. 2013). As a homeostatic mechanism, the UPR alleviates ER stress by two primary intracellular events: 1) Decreasing the demand for protein folding by the down-regulation of new protein synthesis; this is followed by: 2) Transcriptional induction of ER-resident molecular chaperone genes that are involved in protein folding, as well as the

activation of the ER-associated degradation (ERAD) system that enhances the degradation of the misfolded proteins (Meusser et al. 2005). The UPR modulates these processes by asserting control at both transcriptional and translational levels. If these processes are not achieved within a certain time or if the disruption is prolonged, UPR signaling eventually induces cell death by apoptosis (Malhotra and Kaufman 2007).

The UPR consists of three main signaling pathways initiated by three distinct ER stress sensors: inositol-requiring protein 1 (IRE1), protein kinase RNA (PKR), (PKR)-like ER kinase (PERK, also known as EIF2AK3), and activating transcription factor 6 (ATF6). They are integral ER membrane proteins that deliver signals from the ER to the cytosol and the nucleus following ER stress. The activation of each of these sensors of the UPR is dictated by their interaction with the ER luminal chaperone, glucose-regulated protein 78 kDa (GRP78; also called BiP) (Bertolotti et al. 2000). GRP78 is critical to the regulation of the ER stress response and considerable redundancy exists between different signaling pathways. Under homeostatic conditions, GRP78 binds the luminal domain of ATF6, IRE1 and PERK, suppressing their activation. During ER stress, GRP78 dissociates from these transmembrane signaling proteins, and preferentially binds to unfolded proteins (Fig 1.2). Dissociation of GRP78 allows sensor oligomerization and thereby initiates the UPR.

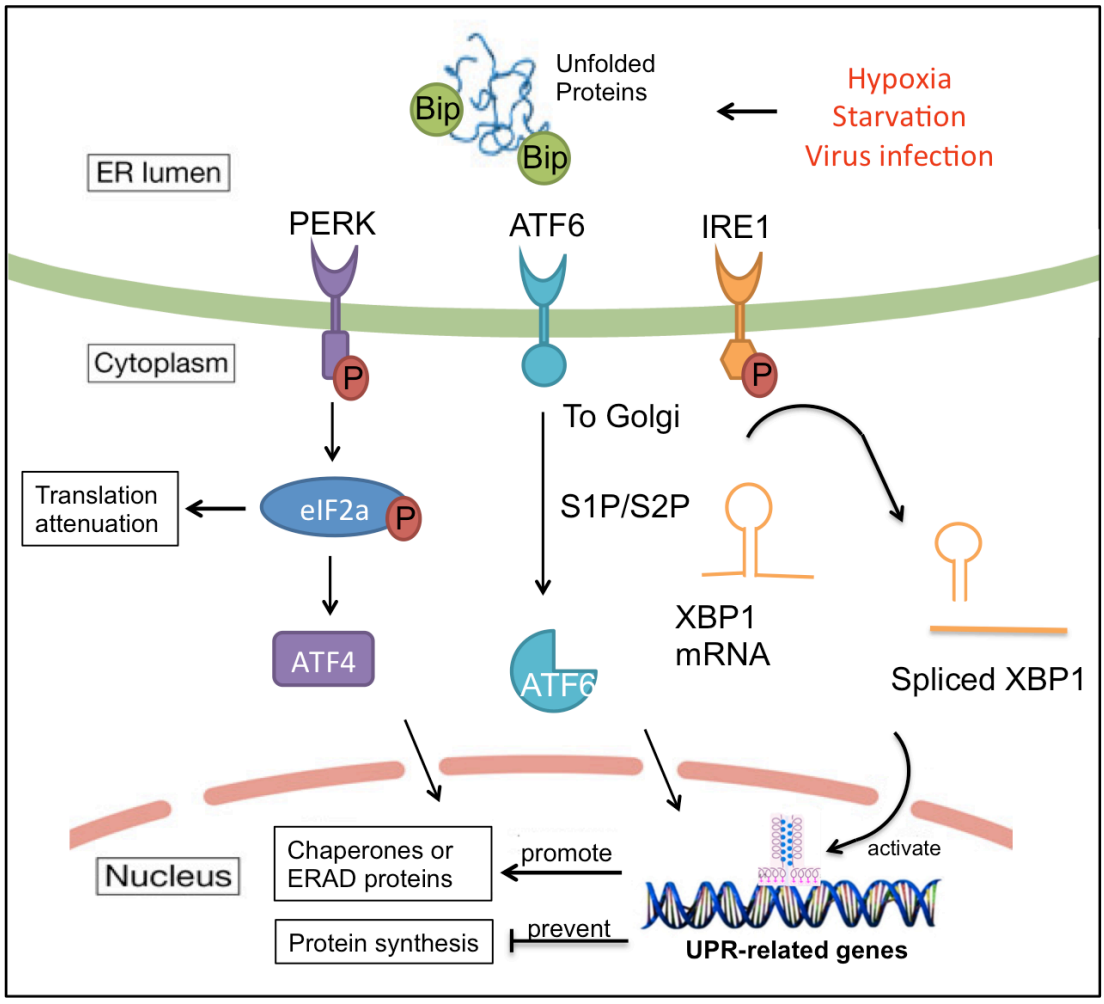

Figure 1.2 The three signaling pathways (IRE1, PERK and ATF6) of the Unfolded Protein Response (UPR). Under homeostatic conditions, GRP78 (also called BiP) binds the luminal domain of ATF6, IRE1 and PERK, suppressing their activation. During ER stress, GRP78 dissociates from these transmembrane signaling proteins, and preferentially binds to unfolded proteins. The dissociation of GRP78 from the three UPR senssors promotes PERK-dependent phosphorylation of eIF2α, which leads to the activation of the transcription factor ATF4. Concomitantly, IRE1 is phosphorylated and activated to mediate the splicing and activation of Xbp1, and ATF6 is proteolytically processed by two Golgi resident enzymes (S1P/S2P). The activated transcription factors ATF4, Xbp1 and ATF6 migrate to nucleus and drive a global transcriptional induction of chaperones and quality control factors that regulate protein systhesis and degradation.

1.3.2.1 IRE1-Xbp1 pathway

Inositol-requiring enzyme 1 (IRE1) is a type I transmembrane serine/threonine protein kinase receptor. The accumulation of unfolded proteins in the ER can induce the oligomerization of IRE1, which further activates Ser/Thr kinase activity of IRE1 and results in the autophosphorylation of IRE1 on Serine residue 724. Phosphorylation of IRE1 activates its mRNA endoribonuclease activation, catalyzing a unique splicing event that generates a shorter spliced form of mRNA encoding X-box-binding protein 1 (Xbp1; HAC1 in yeast) (Fig 1.2) (Calfon et al. 2002). Xbp1 is a transcription factor that belongs to the basic leucine zipper (bLZip) family. Under the normal conditions, Xbp1 exists as an inactive protein with 261 amino acids; once IRE1 is activated during the UPR, an intron of 26 nucleotides of Xbp1 will be spliced by IRE1, creating an active Xbp1 protein. The spliced Xbp1 is a transcription factor that can translocate into the nucleus and activate a variety of UPR relevant genes by binding to the UPR promoter element (UPRE). These target genes are usually required for protein folding and modification, ER-Golgi transport, and ER-assisted degradation (ERAD) (Yoshida et al. 2001; Schroder and Kaufman 2005; Yamamoto et al. 2007).

1.3.2.2 PERK/eIF2α/ATF4 pathway

PERK is also a type I transmembrane Ser/Thr protein kinase receptor, the catalytic domain of which shares substantial homology to other eukaryotic initiation factor 2α (eIF2α) family kinases. Dissociation of GRP78 (also known as Bip) from PERK in ER membranes induces its auto-phosphorylation and kinase domain activation, resulting in phosphorylation of serine-51 of eIF2α in the cytosol (Fig 1.2). eIF2α phosphorylation shuts off mRNA translation, leading to a general inhibition of global protein synthesis, thereby indirectly inhibiting the accumulation of toxic misfolded proteins and reducing the protein load on the ER (Schroder and Kaufman 2005). In addition, phosphorylated eIF2α also mediates the specific and selective translation of certain mRNAs, including the mRNA encoding transcription factor ATF4. ATF4 is translated by a special mechanism controlled by two upstream open reading frames (uORF): uORF1 and uORF2 (Fig 1.3). The 5'-proximal uORF1 is a positive-acting element that always gets translated

first, then facilitates the 40S ribosomal subunit to reinitiate translation at a downstream start codon, depending on the levels of eukaryotic initiation factor (eIF) 2-GTP. Under non-stressful conditions, the phosphorylated eIF2α (eIF2α-P) is low and eIF2-GTP levels are abundant to bind to methionyl-tRNA (Met-tRNA), allowing the ribosome to readily acquire the eIF2-GTP-Met-tRNA complex and reinitiate translation of uORF2. The uORF2 overlaps with the coding sequence of ATF4 and prevents its translation. Once the uORF2 is translated, the ribosome will dissociate from the ATF4 mRNA. However, under the ER stress, the level of eIF2-GTP is low with an increase in eIF2α-P. As a consequence, the 40S ribosome needs more time to reacquire the eIF2-GTP-Met-tRNA complex, allowing it to scan through the uORF2 initiation codon and subsequently obtain the limiting eIF2-GTP-Met-tRNA complex and translate the ATF4 (Fig 1.3). The ATF4 protein is a member of the bLZip family of transcription factors, which contributes to the regulation of a variety of genes involved in amino acid metabolism and transport, antioxidative stress responses, and ER chaperone synthesis (Bernales et al. 2006). These target genes can further increase the levels of chaperones, restore cellular redox homeostasis, and help the ER to either fold proteins or degrade them.

In addition, the PERK pathway also up-regulates a pro-apoptotic transcription factor, the C/EBP homologous protein (CHOP/GADD153), under conditions of extensive or prolonged ER stress, which plays an important role in ER stress-induced cell death. CHOP can exacerbate ER stress by increasing the ER load and by inducing the expression of ER oxidase ERO1α, which makes the ER lumen more oxidative (Marciniak et al. 2004). On the other hand, induction of CHOP in turn causes phosphatase growth arrest and DNA damage 35 (GADD34) expression. When interacted with protein phosphatase 1, GADD34 dephosphorylates eIF2a, relieving its inhibitory effects on eIF2β. Thus, protein translation recovers, and a negative feedback loop is completed (Wang et al. 1996).

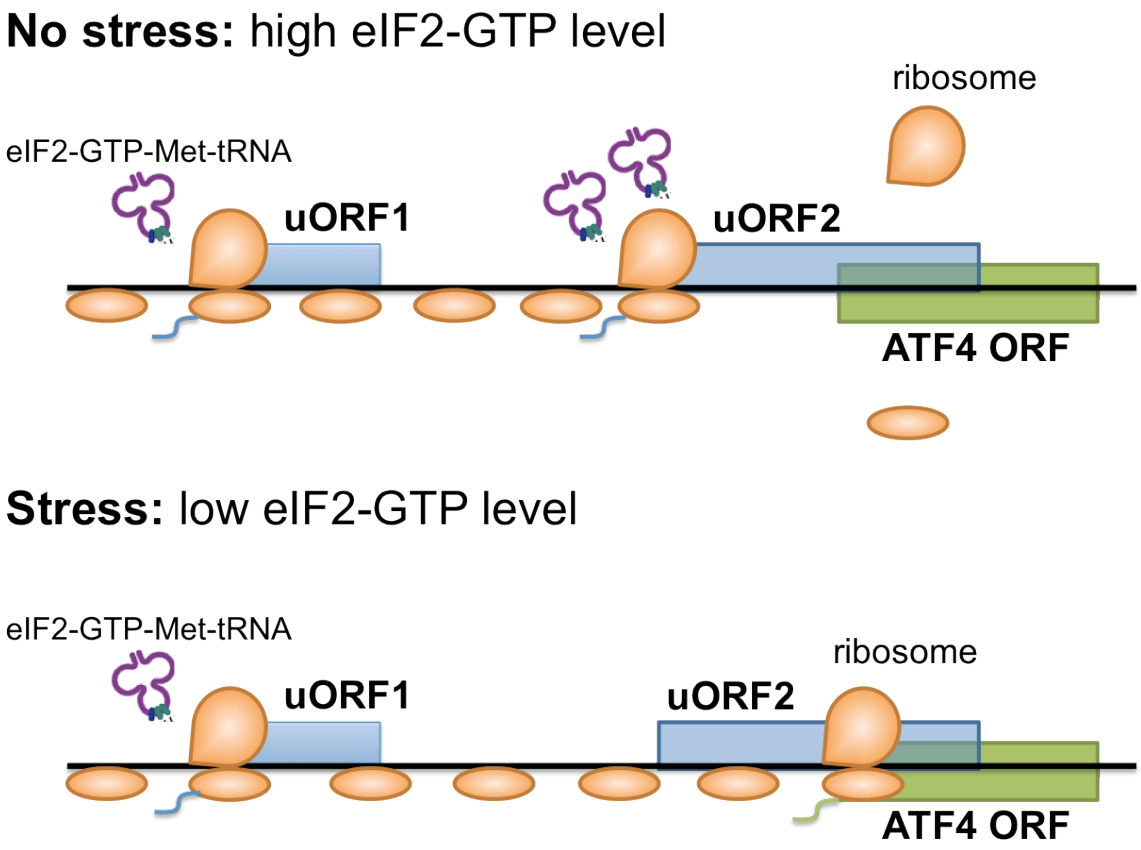

Figure 1.3 Mechanism of ATF4 translation during phosphorylation of the α subunit of eukaryotic initiation factor 2 (eIF2α-P). Under non-stressful conditions, the concentration of phosphorylated eIF2α (eIF2α-P) is low and eIF2-GTP levels are abundant. It then binds methionyl-tRNA (Met-tRNA), allowing the ribosome to readily acquire the eIF2-GTP-Met-tRNA complex and initiate translation of uORF2. The uORF2 overlaps with the coding sequence of ATF4 and prevents its translation. Once the uORF2 is translated, the ribosome will dissociate from the ATF4 mRNA. When the ER is stressed eIF2a is phosphorylated by PERK (see Fig 1.2). This leads to a decrease in levels of eIF2-GTP and therefore the eIF2-GTP-Met-tRNA complex. As a consequence, the 40S ribosome needs more time to reacquire the eIF2-GTP-Met-tRNA complex, allowing it to scan through the uORF2 initiation codon and subsequently obtain the limiting eIF2-GTP-Met-tRNA complex and translate the ATF4.

1.3.2.3 ATF6 pathway

Activating transcription factor 6 (ATF6) is also an ER-localized transmembrane protein, which includes two isoforms (ATF6α and ATF6β). ER stress triggers a different mechanism of protein activation for ATF6 proteins compared with PERK and IRE1. Instead of oligomerization, activation of ATF6 involves regulated intramembrane proteolysis, which liberates the cytoplasmic portion of ATF6 from the ER membrane under conditions of ER stress (Haze et al. 1999). The release of ATF6 proteins from GRP78 allows them to translocate to the Golgi apparatus, where the full-length 90-kDa ATF6 is proteolytically processed by two Golgi resident enzymes: site-1 protease (S1P) and site-2 protease (S2P), thus releasing a 50-kDa cytosolic basic leucine zipper (bLZip) transcription factor (Haze et al. 1999). The cleaved ATF6 migrates into the nucleus and binds to several different promoter elements to promote the transcription of ER stress-related genes (Fig 1.2). The target genes of the ATF6 pathway that have been identified include genes encoding CHOP, ER-resident chaperones (e.g., GRP78, GRP94, PDI, among others), ERAD components, and ER degradation-enhancing α-mannosidase-like protein 1 (EDEM1) (Yamamoto et al. 2007), resulting in increased ER chaperone activity and degradation of misfolded proteins.

1.3.3 UPR and cancer

The importance of the UPR in cancer development is becoming increasing clear. Rapid growth of tumour cells coupled with inadequate vascularization often leads to hypoxia, nutrient deprivation, and metabolic dysregulation that cause ER stress and the UPR. Under the mild to modest stresses, the adaptive and anti-apoptotic pathways of the UPR are activated to protect tumour cells from apoptosis, and to allow them to survive, migrate and escape therapy in unfriendly tumour microenvironment.

Accumulating evidence has demonstrated that the UPR pathways are activated in several tumour types, including the overexpression of Xbp1, phosphorylation of eIF-2α, induction of ATF4 and CHOP, activation of ATF6, and up-regulation of ER chaperones (reviewed by (Davenport et al. 2008; Li et al. 2011). Therefore, the repression of UPR pathways may direct an alternative strategy for cancer therapy.

1.4 p53 signaling pathways and cancer therapy

1.4.1 Structure and function of p53

p53 is a major tumour suppressor protein that is essential for the prevention of cancer development. The human p53 gene is located on chromosome 17 (17p13) and codes for a protein of 393 amino acid residues comprising several functional domains (Joerger and Fersht 2008) as described (Fig 1.4):

- The transactivation domain (TAD) localizes in the amino-terminus part of p53 and is responsible for binding to and activating various downstream target proteins with high sequence-specificity.
- The proline-rich region (PRR) contains five PXXP motifs, which mediate protein-protein interactions in signal transductions as well as response to DNA damage through apoptosis.
- The core DNA binding domain of p53 (p53 C) consists of a multitude of structural motifs that provide the basic scaffold for the DNA-binding surfaces. Its importance is reflected by the high rate (>90%) of p53 mutations in human cancers found in this domain, where even a single mutation can cause major conformational changes and interfere with the DNA-binding ability of the protein.
- The tetramerization domain (TET) consists of a β-strand that mediates the formation of the quaternary structure of p53, which is important for functional DNA binding and p53 affinity to specific DNA.
- The C terminus (CT) hosts the nuclear localization sequence of p53, including three putative nuclear localization signals (NLS) and two putative nuclear export signals (NES).

Figure 1.4 Structure domains of p53 protein. The p53 protein consists of an N-terminal transactivation domain (TAD, 1-60 amino acids), a proline-rich region (PRR, 62-97 aa), the core DNA binding domain (p53 C, 102-292 aa), the tetramerization domain (TET, 320-356 aa) and the C terminus (CT, 363-393 aa). There are several nuclear localization signals (NLS) and nuclear export signals (NES) in the C terminus of p53.

1.4.2 p53-mediated cell cycle arrest and apoptosis

In normal cells, p53 is a short-lived protein and functions to control excessive cell proliferation. Low intranuclear concentrations of p53 protein are maintained by its binding to the E3 ubiquitin-ligase mdm2, which keeps p53 in check by continuous ubiquitylation and subsequent degradation by the 26S Proteasome.

When the cell is stressed by DNA damage, hypoxia, cytokines, metabolic changes, viral infection, or oncogenes, the activation of p53 can trigger cell cycle arrest, thereby providing time for DNA repair before replicating the genome. Hence, p53 prevents the transmission of damaged genetic information from one cell generation to the next. If the damage is too severe to be repaired, p53 will initiate cell apoptosis and work as an emergency brake on cancer development by killing cells that attempt to proliferate in harsh microenvironments, such as those caused by hypoxia, nutrient deprivation, UV radiation and chemotherapy.

p53 mainly functions as a transcription factor. Under the stress conditions, p53 becomes phosphorylated on multiple sites, such as Ser15, Thr18, Ser 20 or Ser46. A potential outcome of such phosphorylation is the stabilization of p53 through disruption of mdm2 association and inhibition of p53 ubiquitination and degradation. Once activated, p53 represses or enhances transcription of a variety of target genes involved in DNA repair,

cell cycle control and/or apoptosis. These include p21, PIG3, NOTCH1, E2F, bax and PUMA. p21 for example, functions as an important inhibitor of a number of cyclin-dependent kinases (CDKs) that are active in the late G1, S, G2 and M phases of the cell cycle. Thus, by stimulating the transcription of the p21 gene, p53 prevents cell proliferation at many points of cell cycle (Amaral et al. 2010; Wei et al. 2012).

p53 initiates apoptosis directly (through interaction of the Bax) or indirectly (through transcription of genes involved in apoptosis). Once p53 becomes activated, a wide network of apoptotic signals, such as Bax, the Fas receptor, and the IGFBP3 protein, are activated and expressed. These signals act through two major apoptotic pathways: intrinsic pathways and extrinsic pathways (Fig 1.5).

The intrinsic apoptotic pathway is controlled by the proteins involved in Bcl2 family, which govern the release of Cytochrome-C (CytoC) and other pro-apoptotic proteins from the mitochondria. Interestingly, p53 can target and promote expression of genes encoding a key subset of Bcl2 family proteins, including Bax, Noxa, and PUMA. These proteins work to open the mitochondrial channels and release CytoC, which activates the apoptotic pathway by activating Caspases (Caspase-9, 3, 6 and 7).

In addition, an alternative route of p53 to trigger apoptosis is initiated outside the cell by activating pro-apoptotic cell surface receptors, such as the Fas receptor and IGFBP-3. p53 induces the transcription of these ‘death’ receptors, leading to a cascade of activation of Caspases (Caspase8 and Caspase3), which in turn induce apoptosis (Amaral et al. 2009; Sharp et al. 2010; Ozaki and Nakagawara 2011).

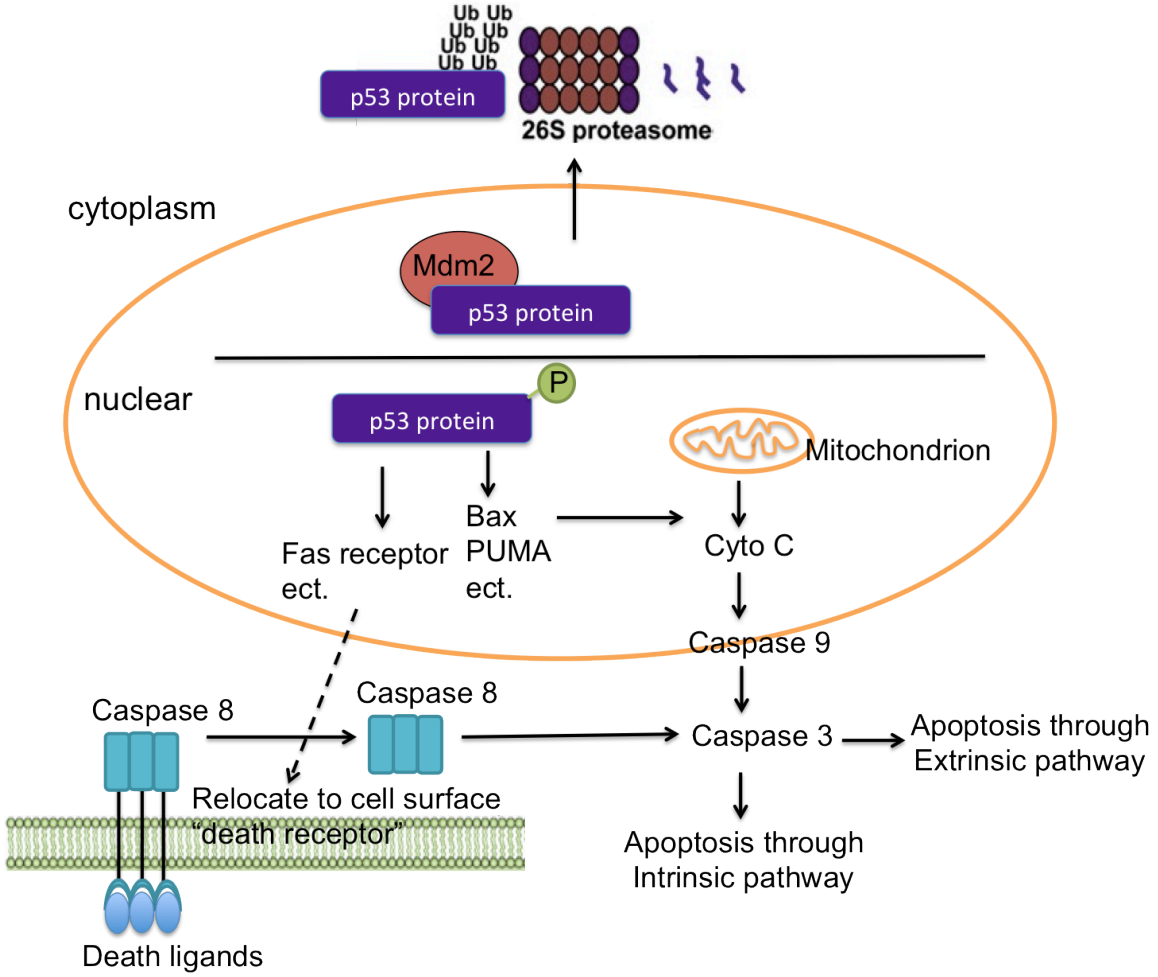

Figure 1.5 Schematic representation of the p53-dependent apoptotic pathways (Intrinsic and Extrinsic pathways). Under normal conditions, the intranuclear concentration of p53 is tightly controlled by its negative regulator mdm2, which binds to p53 and translocates it out of the nucleus for preteasomal degradation via the ubiquitin-dependent pathways. Once p53 is phosphorylated and activated, it induces the expression of proteins that target both the mitochondrial-induced apoptotic pathways (intrinsic pathway) and the death-receptor-induced apoptotic pathways (extrinsic pathway).

1.4.3 p53 mutation and cancer

Given the important role of p53 in tumour suppression, tumour development is often accompanied by high rate of mutations or deletions of the p53 gene, with >50% of human tumours exhibiting some impairment of p53 function. Compromised p53 activity promotes the accumulation of DNA damage in cells, which increases the possibility for malignant transformation (Petitjean et al. 2007). In many cancers containing wild-type p53, the p53 signaling pathways can be altered by other oncogenic events, suggesting that a decrease in p53 function may be a common feature in many cancers.

The fast growth rate and stressful microenvironment of most tumours lead to physiological stresses which would normally lead to p53-triggered apoptosis. However, selection of cells with impaired p53 function during the early phases of tumour development permits these cells to survive under stress conditions that usually lead to the death of normal cells and accumulate mutations at higher rates. This in turn increases the chances of oncogene activation and tumour suppressor gene inactivation.

p53 mutations mainly include the germinal mutation and somatic mutation:

- Germinal mutation:

Germinal mutation of p53 gene causes Li-Fraumeni syndrome, which is an extremely rare inherited disease that greatly increases susceptibility to sarcomas (including osteosarcoma), leukemia and breast cancer. The mutations can be inherited, or can arise de novo early in embryogenesis or in one of the parent's germ cells (Varley 2003).

- Somatic mutation:

Somatic mutations in the p53 gene are one of the most frequent alterations in cancers. More than half of human cancers of breast, bone, colon, lung, liver, prostate, bladder, and skin have the mutated p53 gene. Over 90% of all known tumorigenic mutations are located in the DNA-binding domain, thus preventing p53 from activating the transcription of its target genes. These p53 mutants have different effects on tumour development. One of outcomes of p53 mutations is the loss of tumour suppressor

functions of the wild-type protein, characteristically manifested as a total lack of p53 expression or production of unstable or truncated mutant proteins. On the other hand, the cancer-associated p53 mutations also endow the mutant protein with new activities that can contribute actively to various stages of tumour progression and to increased resistance to anticancer treatments. These activities are referred to as mutant p53 gain-of-function (GOF) (Oren and Rotter 2010). For example, transfection of p53-null cells with mutant p53 enhanced tumour formation in mice (Wolf et al. 1984).

1.4.4 Activation of p53 as a therapeutic strategy

Since p53 activation induces apoptosis in response to physiologic stresses, such as DNA damage, hypoxia, cytokines, metabolic changes, viral infection, or oncogene signaling, there are several therapeutic drugs that might be used in cancer therapy.

1.4.4.1 DNA-damaging agents in cancer chemotherapy

Many chemotherapeutic drugs are DNA-damaging agents that can activate p53-mediated apoptosis in cancers. However, studies have shown that the ability of many DNA-damaging agents to induce p53 is not due to their ability to damage DNA. For many of these compounds, p53 is activated through the inhibition of transcription that results in nucleolar disruption. Nucleolar disruption is related to defects in ribosome biogenesis and further leads to the release of free ribosomal proteins. These proteins can bind to mdm2 and inhibit its interaction with p53, thus activating the p53 response (Zhang and Lu 2009; Lane et al. 2010). The agents that activate p53 through this route include the CDK inhibitors, ribonucleotide production inhibitors and the RNA polymerase inhibitors (reviewed by (Lane et al. 2010).

1.4.4.2 Gene therapy-based approaches

1.4.4.2.1 Introduction of wild-type p53 in tumours

The re-introduction of the wild-type p53 gene into a variety of human tumour cells has been shown to induce apoptosis and growth inhibition. Especially in the p53-defective tumour cells, the expression of wild-type p53 has a profound antitumour activity. The

first p53-based gene therapy was performed by injecting a retroviral vector containing the wild-type p53 gene into tumours of patients with lung cancer (Roth et al. 1996). Then, several virus delivery systems were established for p53 gene therapy; the most commonly used was adenovirus. The obvious problems with this approach are the control of the level of p53 expression and the effective delivering of the p53 gene to tumour cells.

1.4.4.2.2 Introduction of siRNA against the negative regulator of p53 in tumours

The overexpression of p53-negative regulatory proteins in tumour cells may result in the inactivation of wild-type p53, so siRNA can be used against these proteins to activate p53 response. For example, in tumours where p53 is inactivated by ubiquitin-E3 ligase mdm2, the introduction of siRNA specific to mdm2 induced an effective p53 response (Jiang and Milner 2002).

1.4.4.3 Reactivation of mutant p53

Because of the high frequency of p53 mutation in tumour cells, the restoration of p53 has been considered an attractive cancer therapeutic strategy. Some compounds activate p53 response by reactivating the wild-type p53 functions of mutant p53. For instance, the small molecule CP-31398 induces specific p53 response and apoptosis in tumour cells by stabilizing the active conformation of newly synthesized p53. PRIMA-1 (p53 reactivation and induction of massive apoptosis) is another small molecule that restores sequence-specific DNA binding and p53-dependent apoptosis. MIRA-1 (mutant p53-dependent induction of rapid apoptosis) has been shown to reactivate DNA binding and preserve the active conformation and transcriptional function of mutant p53 (Romer et al. 2006; Levesque and Eastman 2007; Lane et al. 2010).

1.4.4.4 Inhibition of nuclear export

p53 is usually inactivated by nuclear export and degradation; thus, the inhibition of nuclear export might be a feasible route to activate p53 response in tumour cells. Crm-1 is an exportin that mediates the transport of proteins from the nucleus to the cytoplasm, while Leptomcin B (LMB) has been known as a potent inhibitor of crm-1. LMB can effectively kill tumour cells in culture by stabilizing p53 from mdm2-mediated

degradation and activating p53-dependent transcription (Lain et al. 1999; Menendez et al. 2003).

1.4.4.5 Inhibition of p53-mdm2 interaction

Mdm2 plays a significant role in the negative regulation of p53. It interacts with p53 and translocates it out of the nucleus for degradation via ubiquitin-dependent pathways. So targeting mdm2 for p53 stabilization with small-molecule antagonists is a promising approach for activating p53. For example, the nutlins act as antagonists of the mdm2-p53 interaction, which can bind in the pocket of mdm2 and prevent it from interacting with p53 (Vassilev et al. 2004).

1.4.4.6 Inhibition of p53 activity after genotoxic stress

Conventional chemotherapeutic agents (such as DNA-damaging agents, antimetaolities, and proteasome inhibitors) and radiotherapy can induce apoptosis following activation of p53 not only in tumour cells, but also in normal cells. Thus, the compound that can inhibit p53 activity is an option to protect normal cells from the severe side effects of cancer treatment, such as the cytoprotective agent Pifihrin-a (Sinn et al. 2010). However, it must be noted that the inhibition of p53 in normal tissue following cancer treatment might increase the incidence of tumours; therefore, the combination of drugs that activate p53 through targeting multiple molecule pathways could be an alternative way to induce apoptosis without genotoxic response on normal and tumour tissues.

1.5 Rationale, Hypothesis and Objectives

One of the main goals of our research group is to determine the role of transcription factor Zhangfei in the growth of cancer cells. In the previous studies, we have demonstrated that Zhangfei dramatically suppressed cell growth in several cancer cell lines, including canine osteosarcoma cells and human medulloblastoma cells (Valderrama et al. 2009; Bergeron et al. 2013). To obtain a clear picture of the functions and characteristics of Zhangfei, I attempted to answer two questions: first, does Zhangfei have a universally inhibitory influence on the growth of different OS cell lines, especially

the canine and human osteosarcoma cell lines? Second, what are the molecular mechanisms by which Zhangfei suppresses the growth of OS cells?

Based on the following observations:

- Zhangfei suppresses cell growth and induces apoptosis in several tumour cells (Valderrama et al. 2009).
- The UPR is an adaptive stress response activated in cancer cells, allowing tumours to survive, develop, metastasize and escape therapy.
- Zhangfei possesses a bLZip motif that exhibits sequence homology with the UPR-related bLZip transcription factors Xbp1, ATF4 and ATF6. Zhangfei can interact with ATF4 through the bLZip region (Hogan et al. 2006).
- Luman/CREB3 is a transcription factor that is structurally similar to and closely related to UPR mediator ATF6 (Raggo et al. 2002).
- Zhangfei suppresses the ability of Luman/CREB3 to activate gene expression (Misra et al. 2005).

My first hypothesis was:

Zhangfei interacts with the UPR-related bLZip transcription factors and inhibits their ability to activate the UPR signaling pathways, thereby suppressing the growth of cancer cells and increasing their susceptibility to ER stressors. Since our laboratory had previously shown that Zhangfei does not have a very suppressive effect on ATF6, I concentrated my efforts on Zhangfei-Xbp1 interactions.

My objectives were to:

- Determine the effect of Zhangfei on the growth and differentiation of several canine and human tumour cell lines.
- Examine the effect of Zhangfei on the UPR signaling pathways and determine if Zhangfei interacts with the UPR-related bLZip transcription factor Xbp1.
- Determine the molecular mechanisms through which Zhangfei exerts its effects in cancer cells.

I found the following: 1. Zhangfei suppressed the UPR and growth of some cell lines, but had no effect or only had marginal effects on others (Chapter 2); 2. Zhangfei inhibited the UPR, at least partially, by binding to the UPR-mediator, Xbp1, and targeting it for proteasomal degradation (Chapter 3). Since the effect of Zhangfei on OS cells was not universal—some cells lines were affected while others were not—my results suggested that sensitivity or resistance to Zhangfei was an inherent property of OS cell lines.

I, therefore, developed another hypothesis based on the following observations:

- The suppressive effects of Zhangfei on cell growth and the UPR are not universal, and it has no obvious effects on untransformed cells and some cancer cell lines.
- Tumour suppressor protein p53 plays a pivotal role in cell cycle arrest and apoptosis of cancer cells, and its expression is tightly controlled in normal cells.
- Tumour development is often accompanied by mutation or loss of p53.
- Zhangfei is a positive regulator of p53 (Lopez-Mateo et al. 2012).

My second hypothesis was that:

Zhangfei mediates its effect on cell growth and the UPR through an intermediary, p53, that is either not induced or is defective in cells that are not affected by Zhangfei.

My objectives were to:

- Examine the effect of Zhangfei on p53 pathways in Zhangfei-responsive and unresponsive cells.
- Determine if Zhangfei suppresses cell growth and the UPR through p53.
- Characterize the interaction between Zhangfei and p53 in the regulation of cancer cell growth.

I demonstrated that Zhangfei, at least in part, suppressed cell growth and the UPR in osteosarcoma cells through direct interaction with tumour suppressor protein p53. Zhangfei was found to stabilize p53 and promote its nuclear retention by displacing the E3 ubiquitin ligase, mdm2 (Chapter 4 and Chapter 5).

2. The Effect of Zhangfei on the Unfolded Protein Response (UPR) and Growth of Cells Derived from Canine and Human Osteosarcomas

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This Chapter determined the effects of transcription factor Zhangfei on the growth and the UPR of canine and human osteosarcoma cell lines. The manuscript "The Effect of Zhangfei on the Unfolded Protein Response and Growth of Cells Derived from Canine and Human Osteosarcomas" has been published in *Veterinary and Comparative Oncology* by Bergeron, T., Zhang, R., et al. 2013. 11(2):140-150 and is reproduced here with the permission of the copyright owner.

My contributions to this manuscript: I examined the effects of Zhangfei on the growth and programmed cell death (apoptosis) of canine and human osteosarcoma cell lines by cell proliferation assay and Annexin V Apoptosis detection, respectively (Fig 2.5). In addition, I also performed the immunoblotting detection to determine that the suppressive effect of Zhangfei on transcripts for UPR-related genes was reflected in a decrease in UPR proteins as well as in cells treated with calcium ionophore thapsigagin (Fig 2.4). Bergeron, T., and others performed the rest of the experiments and obtained the data in Fig 2.1-2.3 and Fig 2.5D.

2.1 Abstract

The objective of this study was to determine whether the protein Zhangfei could suppress the unfolded protein response (UPR) and growth of osteosarcoma cells. Dog (D-17) and a human (Saos-2) osteosarcoma cells were infected with adenovirus vectors expressing either Zhangfei or the control protein β-galactosidase. We monitored the growth rate of the cells by using the WST-1 cell proliferation reagent and designed oligonucleotide primers that could be used to measure relative levels of transcripts for selected UPR genes from dogs and humans. Two of the down-stream UPR proteins—HERP and GRP78—were detected by immunoblots. The mechanism of programmed cell death was determined by monitoring cells for the markers of apoptosis, autophagy and macropinocytosis. We found that Zhangfei suppressed the growth of both D-17 and Saos-2 cells. Zhangfei-expressing D-17 cells displayed large vacuoles containing pinocytosed culture medium and expressed phosphatidylserine on their external surface suggesting that Zhangfei induced macropinocytosis and apoptosis in these cells. While Zhangfei inhibited the growth of both D-17 and Saos-2 cells, it inhibited thapsigargin-induced UPR, as detected by a decrease in transcripts for UPR genes, and HERP and GRP78 proteins, only in D-17 cells, suggesting that the ability of Zhangfei to suppress the UPR and tumour cells growth may not be linked.

Key words: unfolded protein response, osteosarcoma, Zhangfei, CREB-ZF, basic leucine-zipper, apoptosis, macropinocytosis

2.2 Introduction

The fast growth rate of most cancer cells subjects them to stresses such as hypoxia, lack of nutrients and accumulation of metabolic by-products. These stresses force cells to activate responses that induce angiogenesis, metastasis and resistance to treatment by radiation and chemotherapy (reviewed by (Dewhirst et al. 2008)). Three interconnected signalling pathways regulate cellular response to stress (reviewed by (Wouters and Koritzinsky 2008)). These are: hypoxia inducible family of transcription factors (HIFs), the kinase mammalian target of rapamycin (mTOR) and the unfolded protein response (UPR). Several chemotherapeutic agents are directed towards suppressing HIF (reviewed by (Poon et al. 2009)) and mTOR. However, awareness of the involvement of UPR in the stress response of cancer cells is relatively recent (Feldman et al. 2005) and apart from Geldanamycin and Nelfinavir (an anti-HIV drug), few effective drugs are directed against this pathway.

The UPR is activated by three sensors embedded in the endoplasmic reticulum (ER) of cells. These include inositol requiring enzyme (IRE1), putative receptor protein kinase (PERK) and activation transcription factor (ATF)6. The three sensors, IRE1, PERK and ATF6, are held in a quiescent state until they are activated by a variety of stressors in the ER such as the presence of unfolded proteins, changes in glycosylation of proteins, redox status, glucose availability, calcium homeostasis or hypoxia. IRE1 and PERK, in turn, activate the basic leucine-zipper motif (bLzip) transcription factors—Xbp1 and ATF4. ATF6, which is itself a bLzip transcription factor, is translocated upon activation from the ER, via the Golgi apparatus to the nucleus. Xbp1, ATF4 and ATF6 then activate the expression of genes that relieve ER stress. If they are successful, as yet little understood mechanisms turn them off. If ER stress is not relieved, the cells are directed to commit suicide (apoptosis). The UPR is intimately linked to HIF and mTOR pathways (Wouters and Koritzinsky 2008).

The protein Zhangfei is expressed in mature neurons (Akhova et al. 2005) but is not detected in immature neurons or in cells derived from medulloblastomas and neuroblastomas (Valderrama et al. 2009). Recently, we demonstrated that ectopic

expression of Zhangfei in medulloblastoma cells caused them to cease growth and eventually die (Valderrama et al. 2009). While Zhangfei possesses a bLzip motif, its basic domain lacks an asparagine residue that in other bLzip transcription factors is considered crucial to their ability to recognize response elements in gene promoters. Likely as a consequence of this, Zhangfei, as a homodimer, cannot bind known response elements for bLzip transcription factors (Lu and Misra 2000b). Zhangfei can, however, suppress the ability of some other bLzip transcription factors such as Luman/CREB3—a transcription factor closely related to ATF6—(Misra et al. 2005), as well as Xbp1, ATF4 and ATF6 (our unpublished observations) to activate gene expression.

Many spontaneous dog cancers have similar histological characteristics, behaviour and genetics to their human counterparts and respond in similar ways to therapeutics. Spontaneous dog tumours therefore provide opportunities for studying signalling pathways that regulate canine and human tumour growth and for the development of therapeutic agents that might prevent their growth (reviewed by (Mueller et al. 2007; Paoloni and Khanna 2008; Khanna et al. 2009). There is little information on the role of ER stress in canine cancers although Klopfleisch and colleagues have recently shown that metastatic and intra-lymphatic canine mammary tumour cells contain higher levels of Derlin-1, a component of the UPR and a transporter for unfolded proteins from the ER (Klopfleisch et al.). This suggests that the ER is stressed in these cells and that the UPR may aid cells to metastasize.

Since the UPR enhances the survival of some tumours (Dong et al. 2008; Adham and Coomber 2009; Ni et al. 2009; Romero-Ramirez et al. 2009) the purpose of this study was to develop techniques to monitor the UPR in dogs and humans and to test the hypothesis that the ectopic expression of Zhangfei in cell lines derived from dog and human osteosarcomas would suppress their ability to activate the UPR and, consequently, inhibit their growth. We found that Zhangfei could dramatically inhibit the growth of both dog and human osteosarcoma cells. However, while thapsigargin-induced UPR was suppressed in dog osteosarcoma cells, Zhangfei had relatively little effect on the pathway in human osteosarcoma cells. This suggested that the two effects of Zhangfei—the suppression of the growth of tumour cells and suppression of the UPR may not be linked.

2.3 Materials and Methods

2.3.1 Cell Culture

The dog osteosarcoma cell line, D-17, and the human osteosarcoma cell line, Saos-2, were obtained from the American Type Culture Collection. The human osteosarcoma cell line U-2 OS was from Dr James Smiley (University of Alberta). D-17 cells were grown in Eagles minimal essential medium (MEM) containing 10% foetal bovine serum (FBS), Saos-2 cells in McCoy's medium with 15% FBS and U-2OS cells in Dulbecco's MEM containing Glutamax and 10% FBS. All media contained 100 units each of Penicillin and Streptomycin per ml. Cells were grown at 37^{o}C in an atmosphere of 5% CO_2. All media, FBS and antibiotics were purchased from GIBCO/Invitrogen.

To determine the growth rate of cells in response to various treatments, 1×10^4 cells in 200 µl of medium were plated per well in 96 well culture dishes (Costar, Corning Inc.). Twenty-four hr later cells were either mock infected, infected with adenovirus vectors or were treated with drug or the drug diluent. For an estimate of viable cells in a culture, 20 µl of WST-1 cell proliferation reagent (Roche Applied Science) were added to each well in a set and absorbance at 420 nm measured after one hr of incubation. Cell estimates using WST-1 were repeated at 1, 2 and 3 days after infection or treatment. Each experiment was repeated at least twice and the results were analyzed using non-parametric Kruskal-Wallis and Mann-Whitney tests.

To activate the UPR, cells were treated with 100 nM thapsigargin (Sigma Life Science) for 4 hr. Stock solution of thapsigargin was 750 µM in dimethyl sulphoxide (DMSO). Control cultures were treated with an equivalent amount of DMSO. To determine the effect of Zhangfei on UPR, cells were infected with adenovirus vectors expressing either Zhangfei or beta-galactosidase at a multiplicity of infection of 100 infectious units per cell. Infected cells were treated with thapsigargin one day after infection. Adenovirus vectors (BD Biosciences) were prepared and assayed as described earlier (Misra et al. 2005).

To determine if the cause of death of cells was macropinocytosis, D-17 cells were incubated with 25 μg/ml of Alexa 488 dextran (Molecular Probes/Invitrogen) per ml of medium for 24 hr. After further incubation for 10 min in 10 nM Lysotracker Red (Invitrogen) cells were observed for fluorescence. To test for apoptosis cells were collected by trypsinization and stained with fluorescence isothiocyanate labelled Annexin V and porpidium iodide (Calbiochem). A Cyto-ID autophagy detection kit (ENZO Life Sciences) was used to determine if cells were undergoing autophagy.

2.3.2 Quantitative Real-Time PCR

The sequences of PCR primers used in the study are in Table 1. Primers were designed by aligning coding sequences for mRNA for the dog and human homologues of various relevant genes using CLUSTAL (MacVector 11.1.1, MacVector Inc.) and selecting sequences approximately 300 bases apart, which were identical or almost identical in the dog and human genes. A maximum of two mismatches were tolerated if the two nucleotides at the 3' ends of the primers were conserved. The accession numbers for sequences used for the design of primers are: Xbp1 – XM_849540 (canine), NM_005080 (human), HERP – XM_857875 (canine), DQ837586 (human), CHOP - XM_844109 (canine), NM_004083 (human), BiP/GRP78 – XM_537847 (canine), NM_005347 (human). Figure 1 describes the strategy for the design of primers that discriminated between spliced and unspliced versions of Xbp1.

Table 2.1 Sequence of primers used for qRT-PCR.

Xbp1 spliced	upstream	5'-TCTGCTGAGTCCGCAGCAGG-3'
	downstream	5'-TAAGGAACTGGGTCCTTCT-3'
Xbp1 unspliced	upstream	5'-TCAGACTACGTGCACCTCTGC-3'
	downstream	5'-TAAGGAACTGGGTCCTTCT-3'
HERP	upstream	5'-CCGAGCCTGAGCCCGTCACG-3'
	downstream	5'-CTTTGGAAGCAAGTCCTTGA-3'
CHOP	upstream	5'-TGGAAGCCTGGTATGAGGAC-3'
	downstream	5'-TGCCACTTTCCTCTCGTTCT-3'
GRP78	upstream	5'-GGCTTGGATAAGAGGGAAGG-3'
	downstream	5'-GGTAGAACGGAACAGGTCCA-3'
GAPDH	upstream	5'-TGCCTCCTGCACCACCAACTGC-3'
	downstream	5'-GGGCCATCCACAGTCTTCTGGG-3'

RNA was purified from cells using the RNEasy Plus mini kit with a genomic DNA elimination step (Qiagen) and RNA converted to cDNA with the Quantitect Reverse Transcription kit (Qiagen) using instructions supplied by the manufacturer. Two-step qRT-PCR reactions used a Brilliant SYBR Green QPCR Master Mix (Stratagene) and were performed in a Stratagene MX3005P PCR instrument. Each reaction (25 µl) contained 150 nM of each primer, ROX as a reference dye, Brilliant SYBR Green Master mix and 8 µl of cDNA. The thermal profile for the reactions was: 10 minutes at 95^{o}C, 40 cycles of 30 sec at 95^{o}C, 1 minute at 60^{o}C and 1 minute at 72^{o}C. All primer sets had a reaction efficiency of over 98%. To ensure the fidelity of the reactions, the disassociation profiles for PCR products in all reactions were examined and the PCR products of the first reaction were analyzed by gel electrophoresis and sequence confirmed. Every cDNA sample was also assayed for levels of GAPDH transcripts. Levels of GAPDH were used to normalize the samples. All reactions were analyzed in duplicate and each experiment was repeated at least once and usually twice. Relative fold changes of trancript levels were calculated as 2^{DDCt} where DCt was the threshold value (Ct) for a primer set detecting GAPDH subtracted from the Ct value for a particular target and DDCt was DCt value of sample minus the DCt for control. The veracity of the PCR products was confirmed by electrophoresis through agarose gels and by determining their nucleotide sequence.

2.3.3 Antibodies, Microscopy and Immunofluorescence

Mouse monoclonal antibodies against beta-galactosidase were purchased from Millipore. Antiserum against Zhangfei was prepared in rabbits and has been described (Lu and Misra 2000b). The antibodies have been characterized and are specific for Zhangfei (Akhova et al. 2005). After fixing cells grown on glass coverslips in cold methanol and blocking in phosphate buffered saline (PBS) containing 10% FBS cells were incubated with primary antibodies against beta-galactosidase and Zhangfei. After washing off unbound antibody cells were incubated with goat anti-mouse antibodies labelled with Alexa 488 and goat anti-rabbit antibodies labelled with Alexa 546. After washing coverslips were mounted in PBS containing 10% glycerol and observed using a Zeiss

Axiovert fluorescent microscope. Images were captured using a digital camera attached to the microscope and Northern Eclipse software (EMPIX Imaging Inc).

2.3.4 Detection of proteins by immunoblotting

Procedure was as described in (Akhova et al. 2005). Essentially, cells were lysed in SDS-PAGE buffer and proteins separated by electrophoresis through 10% SDS-polyacrylamide gels. Proteins were then transferred to Hybond-LFP membranes (Amersham, GE Healthcare) using an Amersham MiniVE Electrotransfer unit at 400 mAmps for 90 min. After incubating overnight in 5% BSA in phosphate buffered saline (PBS) the membranes were rinsed with PBS containing 0.1% Tween20 (PBS-T) and incubated in PBS-T containing either rabbit anti-HERP (Abcam), anti-GRP78 (Abcam), anti-ZF (Lu and Misra 2000b) and mouse anti-GAPDH (Chemicon). After washing with PBS-T the membranes were incubated with goat anti-mouse Alexa488 and goat anti-rabbit Cy5 (Amersham). Fluorescent bands were detected and documented using a Typhoon Trio variable mode imager (GE Healthcare).

2.3.5 Detection of apoptotic cells

Cells were collected by trypsinization and stained with Annexin V and propidium iodide using an Annexin V kit (Calbiochem) and instruction supplied by the manufacturer. As a positive control cells were treated with 50 μM etopocide (Calbiochem) for 24 hr. Cells were analyzed in a Coulter EPICS XL flow cytometer.

2.3.6 Statistics

Analyses were performed using IBM-SPSS 19 for the Macintosh. ANOVA and Kruskal Wallis and Mann Whitney tests were used for data with more than two groups. For data with two groups means were compared using an independent sample T-test. Differences with *P* values less than 0.05 were considered significant.

2.4 Results

2.4.1 Detecting the UPR in dog and human osteosarcoma cells

The ER-resident proteins IRE1, PERK and ATF6 sense cellular stress. IRE1, an endoribonuclease, splices mRNA for transcriptionally inactive Xbp1 to a form that is translated into a transcriptionally active factor. PERK suppresses most translation in the cells but activates the translation of the mRNA for the transcription factor ATF4. ATF6, which is itself a transcription factor, is translocated to the Golgi where it is released by intra-membrane proteolysis.

To measure activation of the UPR, we designed qRT-PCR oligonucleotide primers that could selectively detect spliced or unspliced transcripts for Xbp1 (Fig 2.1) as well as primers that could detect representative genes activated by the three bLzip transcription factors—Xbp1 (HERP), ATF4 (CHOP) and ATF6 (GRP78/Bip). To determine if the primers could detect and quantitate the respective transcripts in dog and human cells, we treated cells of dog (D-17) and human (Saos-2) osteosarcoma cells lines with the calcium ionophore thapsigargin, which is known to activate the UPR in many cell-types. The level of transcripts in thapsigargin-treated cells were compared with cells treated with the drug diluent DMSO. To measure spliced and unspliced Xbp1 message, we examined cells 4, 18 and 24 hr after treatment. In both cell types spliced Xbp1 transcripts increased dramatically at 4 hr and then decreased over the next 20 hr (Fig 2.2A). The other UPR transcripts were measured 4 hr after thapsigargin treatment and showed a 10 to 50-fold increase over DMSO-treated cells (Fig 2.2B). The PCR products were of the expected size and sequence of the dog and human gene segments amplified by the primers.

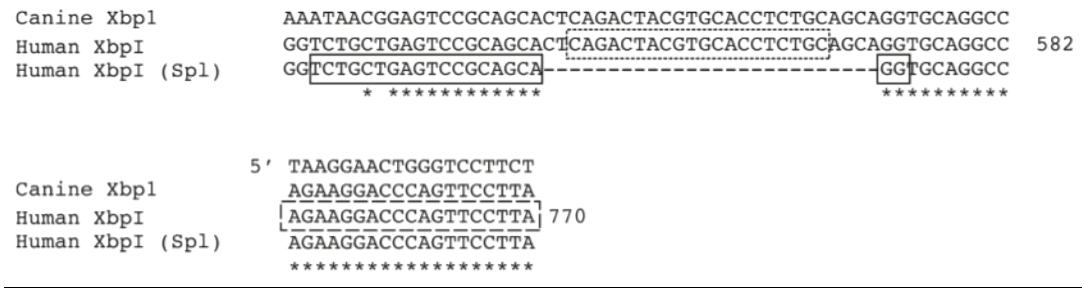

Figure 2.1 Design of primers for detecting and quantifying spliced and unspliced Xbp-1 cDNA. An alignment of coding sequences for canine (XM_849540) and human (NM_005080) unspliced Xbp-1 RNA and spliced human Xbp-1. The numbers indicate nucleotides in NM_005080 and the dashed line the segment removed by IRE1 to generate spliced XbpI mRNA. Sequences selected for primers are boxed—dotted box, upstream primer for unspliced mRNA, the solid box for upstream spliced mRNA and the dashed box for the common downstream primer.

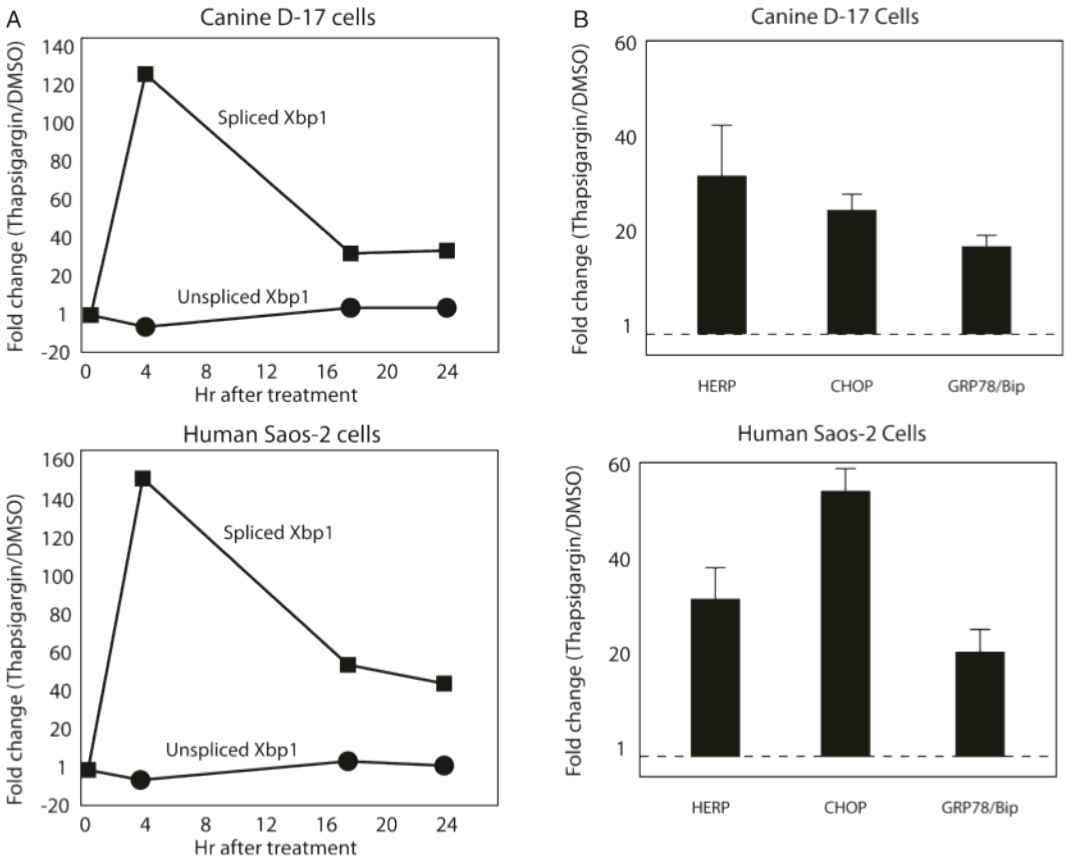

Figure 2.2 The UPR-related transcripts in response to treatment with thapsigargin. **A**. Time course of changes in spliced and unspliced Xbp-1 mRNA in canine D-17 and human Saos-2 cells in response to 100 nM thapsigargin. **B**. Changes in HERP, CHOP and GRP78/Bip transcripts in response to treatment with thapsigargin for 4 hr. Values represent fold changes between Thapsigargin and DMSO (diluent control) treated cells. Bars indicate standard deviation from mean values from three experiments.

2.4.2 Suppression of UPR by Zhangfei

Since Zhangfei can suppress the ability of Luman, an ATF6-like ER-resident transcriptional factor, to activate transcription (Misra et al. 2005), we determined the effect of Zhangfei on the UPR transcripts for Xbp1, HERP, CHOP and GRP78/Bip in thapsigargin-treated cells. Cells were mock-infected or infected with adenovirus vectors expressing either Zhangfei or, as a control, the *Escherichia coli* protein beta-galactosidase (LacZ). One day after infection, cells were treated with either DMSO or thapsigargin, harvested 4 hr later and analyzed for UPR gene transcripts. While LacZ had no obvious effect on the ability of thapsigagrin to activate HERP, CHOP or GRP78/Bip, in Zhangfei-expressing D-17 cells levels of these transcripts were decreased dramatically. Levels of spliced Xbp1 transcripts were also decreased (Fig 2.3, top panel). In contrast, with the exception of a slight decrease in CHOP transcripts, Zhangfei had little obvious effect on level of the UPR transcripts in thapsigargin-treated Saos-2 cells (Fig 2.3, bottom panel).

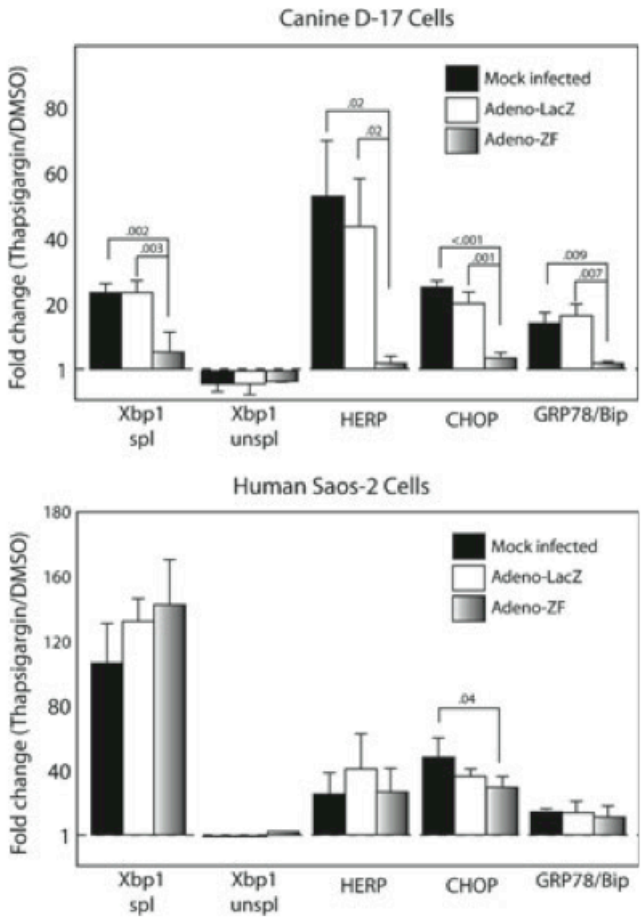

Figure 2.3 Effect of Zhangfei or LacZ-expression on thapsigargin-induced changes in Xbp-1, HERP, CHOP and GRP78/Bip transcript levels in canine D-17 and human Saos-2 cells. Cells were mock-infected or infected with adenoviruses expressing either LacZ or Zhangfei. Twenty four hr after infection cells were treated with either DMSO or thapsigargin. Four hr later cells were harvested and differences in levels of the transcripts in thapsigargin and DMSO-treated (diluent control) cells was assessed. Standard deviations from mean are shown and *P* values are noted above the bars.

2.4.3 Effect of Zhangfei on UPR related proteins

Our results suggested that Zhangfei reduced transcripts for UPR-related genes in cells in response to thapsigargin (Fig 2.3). To determine if this decrease in RNA was reflected in a decrease in UPR proteins as well, we detected HERP and GRP78 in cells mock-infected or infected with an adenovirus vector expressing Zhangfei. We examined cells that expressed Zhangfei for 24 hr and were then treated with thapsigargin for 4 hours. This would determine the ability of cells expressing Zhangfei to respond to the UPR. As an alternative, we treated cells with thapsigargin for 4 hr and then infected or mock infected cells (as in the experiment described in Fig 2.3). This would assess the ability of Zhangfei to turn off the UPR once it had been activated. In both experiments, mock-infected cells were an indication of the level of HERP and GRP78 without Zhangfei at the time of cell harvest. Figure 2.4 shows that cells already expressing Zhangfei (compare lanes 1 and 2 in Fig 2.4) were unable to make detectable amounts of HERP as compared to mock-infected cells. After 4 hr of thapsigargin treatment mock-infected cells made relatively little GRP78 (barely visible as a faint band in lane 2) and even this was not detected in Zhangfei-expressing cells (lane 1). When cells were treated with thapsigargin first, infected or mock-infected, and then maintained in thapsigargin-free medium for 24 hr, substantial amounts of GRP78 were detected in mock-infected cells (lane 4) and this was considerably reduced in Zhangfei–expressing cells (lane 3). Zhangfei also reduced HERP as compared to mock-infected cells (compare faint band in lane 4 with lane 3).

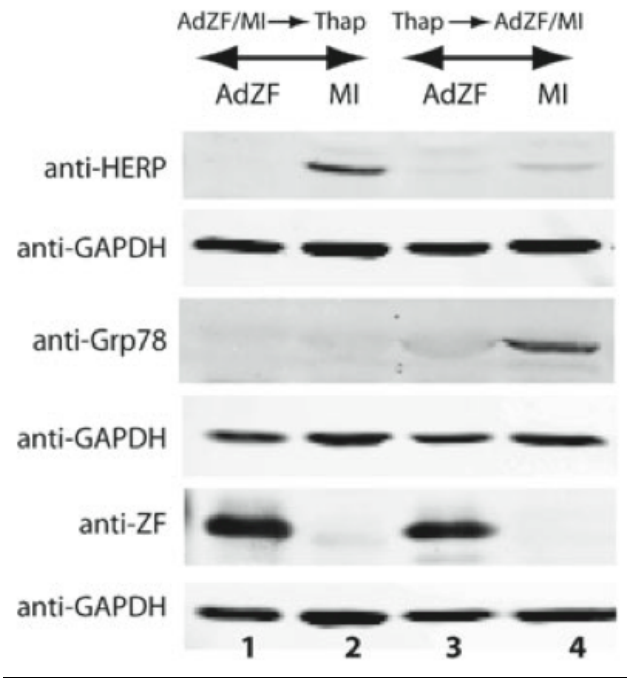

Figure 2.4 Effect of Zhangfei on HERP and GRP78 in cells treated with thapsigargin. D-17 cells were either infected with an adenovirus vector expressing ZF (AdZF) or were mock-infected before (lanes 1,2) or after (lanes 3,4) treatment for 4 hr with thapsigargin. Cells were harvested 24 hr after infection, proteins separated by SDS-PAGE and probed with antibodies against HERP, GRP78 or Zhangfei (ZF) and GAPDH.

2.4.4 Effect of Zhangfei on the growth of dog and human osteosarcoma cells

Ectopic expression of Zhangfei in medulloblastoma cells causes them to stop growing (Valderrama et al. 2009). To determine if Zhangfei had a similar effect on dog and human osteosarcoma cells, D-17 and Saos-2 cells were mock-infected or infected with adenoviruses expressing either Zhangfei or LacZ. Growth was monitored as absorbance at 420 nm of cultures treated for 1 hr with WST-1 cells proliferation reagent. At daily intervals cells were also stained for Zhangfei or LacZ to ensure expression of the proteins in all cells. While expression of LacZ had no effect on the growth of the cells, Zhangfei suppressed the growth of D-17 cells as early as one day after infection (Fig 2.5A). All Zhangfei-expressing cells died approximately 5-6 days after infection while most mock-infected or LacZ-expressing cells retained their viability at that time.

All cells infected to express either LacZ or Zhangfei expressed the proteins and there was little fluorescence because of cross-staining. We noticed that many Zhangfei-expressing D-17 cells showed large vacuoles (Fig 2.5D). Cells die of necrosis or of three known pathways of programmed cell death—apoptosis, autophagy and macropinocytosis (Li et al. 2010). Zhangfei-expressing cells took up large amounts of fluorescent Alexa 488 dextran from the growth medium (Fig 2.5D). Some of the dextran containing vacuoles co-localized with Lysotracker-staining mature lysosomes, a marker of macropinicytosis (Li et al. 2010; Nara et al. 2010). We also determined if Zhangfei induced D-17 cells to commit apoptosis. Intact, unfixed cells were stained with fluorescent Annexin V, 12 and 24 hour after infection. Annexin V binds to phosphatidylserine on the surface of cells, a feature of apoptotic cells. Necrotic cells also bind Annexin V but can be distinguished from apoptotic cells because they are permeable to propidium iodide. As a positive control we analyzed cells treated with etopocide, a chemical that induces apoptosis. Figure 2.5E shows that Zhangfei-expressing D-17 cells began to display markers of apoptosis by 12 hr after infection with adenovirus vector expressing Zhangfei. By 24 hr following infection more than 20% of cells were apoptotic. This compared with about 14% apoptotic cells 24 hr after treatment with etopocide and less than 1% apoptotic cells in mock-infected, Lac-Z-expressing or DMSO-treated (diluent for etopocide) cultures. The proportion of propidium iodide staining cells (dead cells) did not exceed 1% of cells for

any of the samples during 24 hr of infection or treatment. The cells did not display markers of autophagy.

While the effect of Zhangfei on Saos-2 cells was not as dramatic (Fig 2.5B) as on D-17 cells, the growth rate of these cells was significantly different from that of mock-infected ($p=0.023$) and LacZ-expressing cells ($p=0.026$). There was no difference between mock-infected and LacZ-expressing cells ($p=0.956$).

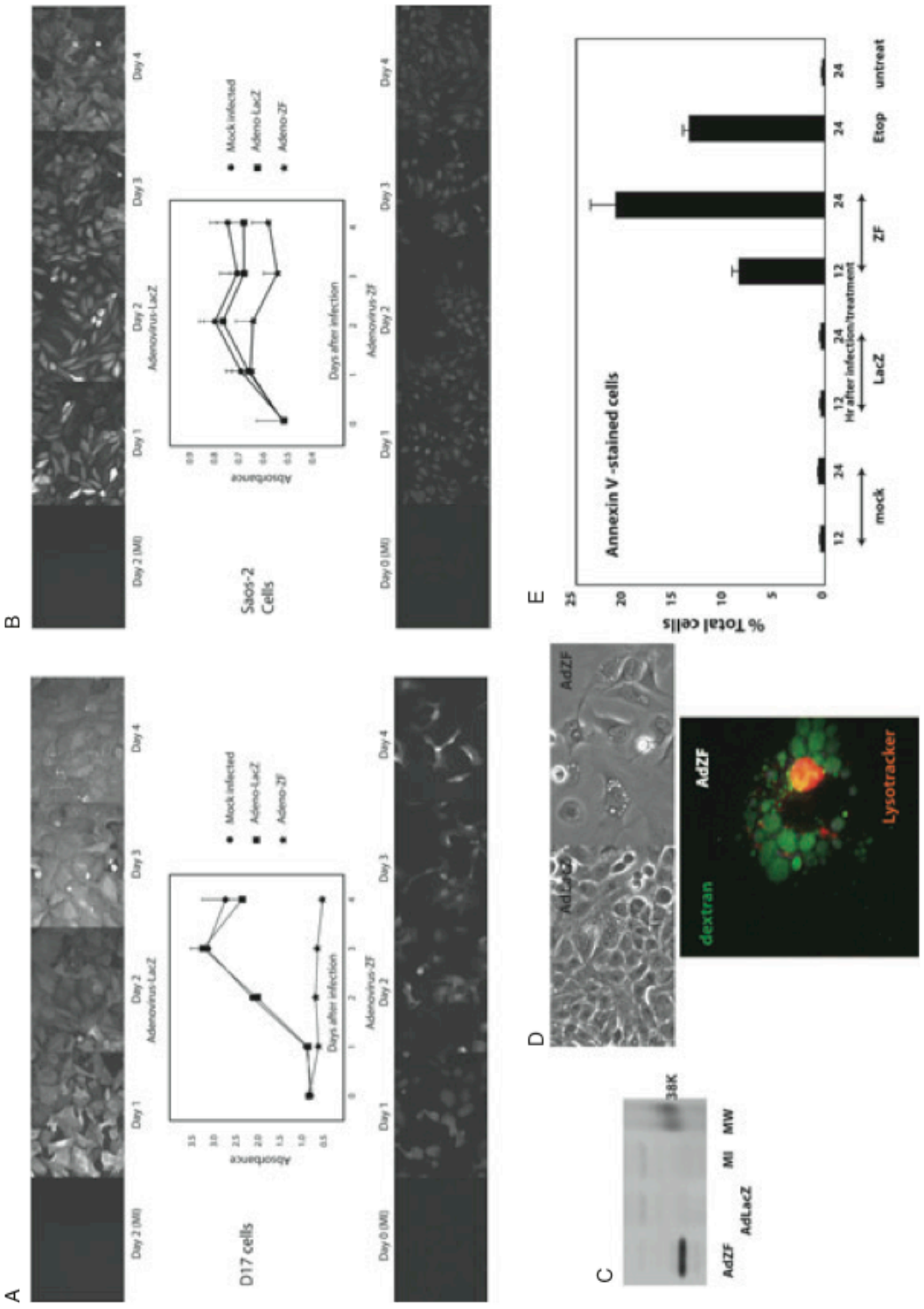

Figure 2.5 Effect of Zhangfei on the growth of canine D-17 and human Saos-2 cells. Cells were mock infected or infected with adenoviruses expressing either Zhangfei or

LacZ. At 24 hr intervals the metabolic activity of cells was assessed by treatment with WST. At daily intervals cells were also fixed and analyzed by immunofluorescence for LacZ (top panel in Fig 2.5A and B) or Zhangfei (bottom panel in Fig 2.5A and B). The difference in the growth rates of Zhangfei and LacZ-expressing D-17 cells is obvious. For Saos-2 cells there was a statistically significant difference between the growth rates of mock-infected or LacZ-expressing cells and Zhangfei-expressing cells, p=0.026 and 0.023, but none between mock-infected and LacZ-expressing cells, p=0.956. (C) Immuno-blot of lysates of D-17 cells mock-infected or infected with Adenovirus vectors expressing LacZ or ZF. The blot was developed with the rabbit antibody against Zhangfei used in Figures A and B. (D) Zhangfei-expressing D-17 stained for macropinosomes (green) and lysosomes (red). Top panel shows phase-contrast micrographs of LacZ and Zhangfei-expressing cells. The cytoplasmic vacuoles in Zhangfei-expressing cells are clearly visible. The lower panel shows Zhangfei-expressing cells grown in medium containing fluorescent (Alexa488, green) dextran and stained for lysosomes (Lysotracker, red). **(E)** AdZF or AdLacZ-infected cells, mock-infected cells and D-17 cells treated with 50μM etopocide were stained with for Annexin V-fluorescence and propidium iodide and cells staining with either or both dyes were enumerated by FACS. Bars represent fraction of cells undergoing apoptosis, ie. percentage of total cells in a sample that stained with Annexin V-fluorescence but not propidium iodide. Where applicable, error bars represent standard deviation from the mean.

2.5 Discussion

Our objective was to determine if Zhangfei could suppress the growth and the UPR in tumour cells and if these two properties attributed to the protein were linked. Our results showed that ectopic expression of Zhangfei could inhibit the growth of dog and human osteosarcoma cells (Fig 2.5). We did not determine the mechanisms by which Zhangfei accomplished this. However, D-17 cells appeared to undergo macropinocytosis and take in large amounts of extracellular fluid (Fig 2.5D). Macropinocytosis is involved in cellular processes such as antigen processing in dendritic cells (Doherty and McMahon 2009) but dysregulation has been shown to cause cell death in glioblastoma cells (Overmeyer et al. 2008) and induction of macropinocytosis by stimulation of the TrkA/NGF pathway causes death of medulloblastoma cells (Li et al. 2010). The inhibition of cell division in human Saos-2 cells was not as dramatic as in D-17 cells and we did not observe the presence of large cytoplasmic vacuoles suggesting that the mechanism by which Zhangfei exerts its effect may be different for the two cell lines.

While Zhangfei dramatically inhibited the ability of thapsigargin to activate markers of the UPR in D-17 cells it had no effect in Saos-2 cells (Fig 2.3) supporting a different mechanism in these two cell lines.

Our results show (Fig 2.4) that the suppression of transcripts for UPR genes by Zhangfei was reflected in its effect on HERP and GRP78 proteins induced by thapsigargin. Zhangfei both prevented the synthesis of the protein in response to thapsigargin and also reduced the proteins in cells in which they had been induced before Zhangfei expression. When comparing the two proteins in mock-infected cells (lanes 2 and 4) we detected more HERP in cells harvested 4 hr after thapsigargin treatment (lane 2) than in cells harvested 24 hr after thapsigargin had been removed (lane 4). In contrast more GRP78 was detected in cells 24 hr after drug treatment (lane 4) than in cells harvested immediately after 4 hr in thapsigargin. This suggests that after thapsigargin treatment HERP is made before GRP78 but then degraded faster than GRP78.

Most bLzip domain-containing proteins regulate transcription as leucine-zipped homo-dimers or as hetero-dimers with other such proteins, the properties of the partners as well

as the DNA motifs they recognize determining whether the dimers activate or suppress gene expression (reviewed in (Miller 2009)). Zhangfei lacks an aspargine residue present in the DNA-binding domain of most bLzip proteins and known to be crucial to their ability to bind DNA (Vinson et al. 1989; Ellenberger et al. 1992). Possibly because of this, Zhangfei does not appear to bind DNA on its own but can activate or suppress gene expression in association with other bLzip proteins (Lu and Misra 2000b). Other studies from our laboratory (unpublished) suggest that Zhangfei suppresses the UPR by binding to and inhibiting the activity of XbpI, ATF4 and ATF6. It was therefore puzzling that it inhibited the UPR in some cells (D-17, Fig 2.3) but not in others (Saos-2, Fig 2.3). This suggests that the mechanism by which Zhangfei suppresses the UPR may be more complex than we had envisioned. An alternative explanation may be that Zhangfei must be post-translationally processed to be active and that the machinery required for this is not present in Saos-2 cells.

Since Zhangfei suppressed growth but not the UPR in Saos-2 cells, our results also suggest that the ability of Zhangfei to suppress the growth of tumour cells may not be linked to its ability to suppress the UPR. Recently we have determined that Zhangfei suppresses the growth of human medulloblastoma cells by activating the expression of TrkA, the high-affinity receptor for Nerve Growth Factor (manuscript submitted). This triggers an autocrine-signalling process that activates genes that lead to differentiation and apoptosis in a mitogen activated protein kinase (MAPK)-dependent process. We have not determined how Zhangfei suppresses other tumours and we are in the process of assessing how many different kinds of human and dog tumours are susceptible to Zhangfei. However, our results thus far suggest that cells obtained from normal tissue may not be inhibited by the protein. This selective targeting of tumour cells is also observed for oncolytic viruses such as reovirus, vesiculo-stomatitis virus and myxovirus which rely on constitutively active components of the MAPK pathway in tumour cells for their selective targeting (Smakman et al. 2006; Wang et al. 2006a; Marcato et al. 2007; Noser et al. 2007).

The selective targeting of the ability of tumour cells to grow as well as the UPR (at least in some tumour cells) may allow the therapeutic use of Zhangfei in combination with other modalities, such as chemotherapy and radiation, that are inhibited by the UPR.

2.6 Acknowledgements

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3. Zhangfei/CREB-ZF – a potential regulator of the Unfolded Protein Response

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This Chapter demonstrated how Zhangfei interacted with the UPR-mediator Xbp1s and consequently suppressed the UPR signaling pathways in cancer cell lines. The manuscript has been published as "Zhangfei/CREB-ZF – a potential regulator of the Unfolded Protein Response" in *PLoS One.* 8(10): e77256. by Zhang, R., Rapin, N., et al. and is reproduced here with the permission of the copyright owner.

My contributions to this manuscript: I designed and performed all the experiments mentioned in this manuscript, except the qRT-PCR arrays and PCR confirmation (Fig 3.1), which were performed by Rapin, N., as well as the adult dorsal root ganglia culture, which was performed by Ying, X.

3.1 Abstract

Cells respond to perturbations in the microenvironment of the endoplasmic reticulum (ER), and to the overloading of its capacity to process secretory and membrane-associate proteins, by activating the Unfolded Protein Response (UPR). Genes that mediate the UPR are regulated by three basic leucine-zipper (bLZip) motif-containing transcription factors—Xbp1s, ATF4 and ATF6. A failure of the UPR to achieve homeostasis and its continued stimulation leads to apoptosis. Mechanisms must therefore exist to turn off the UPR if it successfully restores normalcy. The bLZip protein Zhangfei/CREBZF/SMILE is known to suppress the ability of several, seemingly structurally unrelated, transcription factors. These targets include Luman/CREB3 and CREBH, ER-resident bLZip proteins known to activate the UPR in some cell types. Here we show that Zhangfei had a suppressive effect on most UPR genes activated by the calcium ionophore thapsigargin. This effect was at least partially due to the interaction of Zhangfei with Xbp1s. The leucine zipper of Zhangfei was required for this interaction, which led to the subsequent proteasomal degradation of Xbp1s. Zhangfei suppressed the ability of Xbp1s to activate transcription from a promoter containing unfolded protein response elements and significantly reduced the ability to Xbp1s to activate the UPR as measured by RNA and protein levels of UPR-related genes. Finally, specific suppression of endogenous Zhangfei in thapsigargin-treated primary rat sensory neurons with siRNA directed to Zhangfei transcripts, led to a significant increase in transcripts and proteins of UPR genes, suggesting a potential role for Zhangfei in modulating the UPR.

3.2 Introduction

One of the main functions of the endoplasmic reticulum (ER) is to modify, process and fold proteins destined for secretion or insertion into membranes. The ER also plays critical roles in maintaining intracellular calcium stores, steroid and lipid biosynthesis, membrane regeneration and gluconeogenesis (reviewed in (Fujimoto and Hayashi 2011; Wagner and Moore 2011)). Since protein folding and processing require an oxygen rich microenvironment, as well as adequate nutrient and calcium levels, deficits in these components lead to the accumulation of unfolded or inadequately modified proteins. The ER then initiates a program of recovery called the Unfolded Protein Response (UPR, reviewed in (Merksamer and Papa 2010; Diehl et al. 2011)). The UPR has three main goals: the degradation of misfolded proteins, suppression of the synthesis of new proteins, and to increase the synthesis of chaperones and other proteins required for processing. The suppression of additional protein synthesis is achieved by the phosphorylation of the eukaryotic translation initiation factor 2a (eIF2 a) by the ER-stress sensor—double stranded RNA protein kinase-like ER kinase (PERK) (Harding et al. 1999). In addition, three basic leucine-zipper motif (bLZip) containing proteins: spliced X-box binding protein (Xbp1s), activation transcription factors ATF4 and ATF6, activate the transcription of UPR-related genes. The protein Xbp1s results from the unique extra-nuclear splicing of the mRNA for the transcriptionally inactive protein Xbp1u by the ER stress sensor, inositol-requiring enzyme/ER to nucleus signaling protein (IRE1/ERN1). Xbp1s retains the basic leucine-zipper motif (bLZip) coded by the unspliced Xbp1u mRNA but acquires a transcription activation domain and a nuclear transport motif (Calfon et al. 2002). A failure of the UPR to re-establish normalcy triggers apoptosis while successful homeostasis leads to suppression of the UPR.

The UPR includes feed back mechanisms that mediate a retraction of the UPR if ER function is restored. The proteins GADD34 (Ma and Hendershot 2003; Zhou et al. 2011), Nck1 (Latreille and Larose 2006; Cardin et al. 2007) and p58iPK (Yan et al. 2002; van Huizen et al. 2003) and reviewed by (Rutkowski et al. 2007) recruit protein phosphatases that dephosphorylate eIF2a restoring protein synthesis. The protein Xbp1u, dimerizes with Xbp1s and ATF6 and targets them for proteasomal degradation (Yoshida et al. 2006;

Yoshida et al. 2009). With the exception of Xbp1u, most of the UPR-modulating mechanisms described to date are aimed at the PERK effector pathways of the UPR. Relatively little is known about the suppression of the IRE1 and ATF6 arms of the response.

Zhangfei/CREBZF/SMILE was first discovered as a binding partner for Host Cell Factor (HCF), a co-activator of the herpes simplex virion transcription factor VP16 (Lu and Misra 2000b). Translation for the protein is initiated at two alternate initiation codons (Xie et al. 2008), although both isomers appear to have similar properties. The primary structure of the protein contains a leucine zipper, a basic region that lacks an asparagine residue conserved in most bLZip proteins, three potential nuclear factor binding domains (LLXXLL, where L is a leucine residue and X is any amino acid), and a domain for binding HCF. Zhangfei interacts with several proteins, possibly through its nuclear receptor and HCF binding domains as well as its leucine zipper. While Zhangfei can activate gene expression through factors such as p53 (Lopez-Mateo et al. 2012) and ATF4 (Hogan et al. 2006), it suppresses the activity of a number of transcription factors which include nuclear receptors (Xie et al. 2008; Xie et al. 2009a; Xie et al. 2009b), bLZip containing proteins such as CREBH (Misra et al. 2012) and Luman/CREB3 (Misra et al. 2005), SMAD 1,5,8 (Lee et al. 2012a) and herpes simplex virion associated VP16 (Akhova et al. 2005). We have detected Zhangfei protein in differentiated neurons, but not in developing neurons or cells of neuronal tumours (Akhova et al. 2005). The ectopic expression of Zhangfei in medulloblastomas and other tumours causes the cells to stop growing and eventually to die (Valderrama et al. 2009; Bodnarchuk et al. 2012; Bergeron et al. 2013). Zhangfei suppresses the ability of Luman/CREB3 (Misra et al. 2005) and CREBH (Misra et al. 2012), to activate transcription. Since these proteins are known to regulate the UPR in some cell types, we hypothesized that Zhangfei may be involved in modulating the UPR. Here we show that Zhangfei can suppress the expression of UPR genes activated in response to the drug thapsigargin. We further show that this effect is mediated, at least partially, by the leucine-zipper dependent interaction of Zhangfei and Xbp1s resulting in the proteasomal degradation of Xbp1s. Our results support the hypothesis that Zhangfei has the capacity to modulate the UPR.

3.3 Materials and Methods

3.3.1 Cell Culture

The human medulloblastoma cell line ONS-76 (Tamura et al. 1989), was obtained from Michael Taylor (University of Toronto). These cells were cultured as described previously (Valderrama et al. 2009). Vero cells were obtained from the American Type Tissue Culture Collection and grown in Dulbecco's minimal essential medium containing penicillin and streptomycin and 10% newborn calf serum. All media, serum and antibiotics were purchased from Invitrogen. For some experiments cells were treated with 100 nM thapsigargin or with an equivalent amount of DMSO, the diluent for thapsigargin, for 4 hr. The duration of thapsigargin treatment was based on preliminary experiments to determine the time course of the accumulation of selected UPR-related transcripts following thapsigargin treatment.

3.3.2 Immunofluorescence

Cells were processed for immunofluorescence as described previously (Lu and Misra 2000a). Cells were stained for Zhangfei using anti-FLAG monoclonal antibody (Sigma, 082K9164), for other proteins—rabbit anti-GRP78 serum (Abcam, ab21685), for anti-HERP (Abcam, ab73669-100) and anti-Xbp1 (Abcan, ab37152). Secondary antibodies were Alexa Fluor 488 linked anti-mouse and Alexa Fluor 546 linked anti-rabbit antibodies (Invitrogen, A-11001, A-11035). Cell nuclei were stained using Hoechst fluorescent dye (Molecular Probes, 33342).

3.3.3 Plasmids

The construction of pcZF (Lu and Misra 2000b), a plasmid that expresses Zhangfei in mammalian cells, pCAT3BATF6 (Misra et al. 2005), with the coding sequences for chloramphenicol acetyl transferase (CAT) and pMZF (Lu and Misra 2000b), with the coding sequences for Zhangfei linked to the DNA-binding domain of yeast GAL4, have been described. Plasmid pCGNATF6 (1-373), which expresses the constitutively active truncated form of ATF6, and p5XATF6GL3, which contains 5 copies of the UPRE-containing oligonucleotide, CTCGAGACAGGTGC**TGACGTGG**CATTC, were

obtained from Ron Prywes, Columbia University, USA (Wang et al. 2000). A plasmid expressing the functionally active, spliced form of Xbp1 cDNA was obtained from K. Mori, Kyoto University, Japan (Yoshida et al. 2001). The plasmid, pG5EC, a CAT reporter plasmid with 5 copies of the yeast Gal4-UAS as well as the pM series of plasmids for constructing Gal4 fusion proteins were obtained from I. Sadowski, University of British Columbia, Canada (Sadowski et al. 1992). To construct pcZF(L/A), an expression vector in which the codons for the first six consecutive leucines in the leucine-zipper of Zhangfei were replaced by codons for alanine, a 265 bp synthetic DNA fragment (IDT) bracketed by NotI and SgrA1sites was used to replace a fragment between unique Not1 and SgrA1 sites in the coding sequences of Zhangfei in pcZF. An internal PstI site within the fragment was eliminated with a silent mutation to allow for screening of the mutant. The coding sequences in the mutant were sequenced to confirm the mutation and to ensure that no unintended changes had been made.

3.3.4 Adenovirus vectors expressing Zhangfei and β-galactosidase (LacZ)

These vectors were constructed, grown, and purified using the Adeno-X Expression System (Clontech, K1650-1). They were created in our laboratory as described earlier (Misra et al. 2005). ONS-76 cells were infected with Adeno-Zhangfei, Adeno-LacZ (expressing *E. coli* b-galactosidase, LacZ) or mock-infected. A multiplicity of infection (MOI) of 100 plaque forming units (PFU) per cell was used.

3.3.5 mRNA purification and cDNA synthesis

RNA was purified using RNeasy Plus Mini Kit (Qiagen, 74136) and cDNA synthesized using Quantitect Reverse Transcription Kit (Qiagen, 2053414). One µg of template RNA at a time was converted to cDNA. To ensure high quality RNA for qRT-PCR array analysis, samples were analyzed by electrophoresis on an Agilent 2100 Series Bioanalyzer, Eukaryotic Total RNA Nano series II, version 2.0.

3.3.6 qRT-PCR arrays and PCR confirmation

Unfolded protein response qRT-PCR arrays were purchased (SABiosciences, array number PAHS-089A-12). The array contained primers for 84 gene transcripts involved

in the UPR. The array also contained controls for genomic DNA contamination and reverse transcriptase efficiency. The results from triplicate experiments were analyzed by using a SABiosciences online resource called RT^2 profiler. To confirm the results of the qRT-PCR array we designed primers (Table 3.1) for the activated or repressed genes using human mRNA gene sequences found on the NCBI human genome website (http://www.ncbi.nlm.nih.gov/projects/genome/guide/human/). Sequences were designed using the website tool Primer3 (http://frodo.wi.mit.edu/primer3/) and purchased from Integrated DNA Technologies (IDT). For the normalizer, GAPDH, primers have been described. Agilent Technologies' Brilliant II SYBR Green QPCR Master Mix Kit (catalog number 600828) was used to perform qRT-PCR (in triplicate, also repeated 3 times). The PCR machine used was a Stratagene Mx3005P model. Cycle details are as follows: 95°C for 10 min, followed by 40 cycles (95°C for 30 sec, 55°C for 1 min, 72°C for 1 min) and a final step (95°C for 1 min, 55°C for 30 sec, 95°C for 30 sec).

Table 3.1 Oligonucleotides primers used for Real Time PCR

Gene Name	Symbol	Primer	Derived from (	Sequence	Prod. size (bp)
Activating transcription factor 4	ATF4	ATF4 F	NM_001675	TCA AAC CTC ATG GGT TCT CC	226
		ATF4 R		GTG TCA TCC AAC GTG GTC AG	
X-box binding protein 1	Xbp1	XBP1 F	NM_005080	GGA GTT AAG ACA GCG CTT GG	248
		XBP1 R		ACT GGG TCC AAG TTG TCC AG	
DNA damage inducible transcript 3	DDIT3	DDIT3 F	NM_004083	CTT TCT CCT TCG GGA CAC TG	203
		DDIT3 R		TGT GAC CTC TGC TGG TTC TG	
DnaJ homologue, subfamily B, member 9	DNAJB9	DNAJB9 F	NM_12328	AAA ATA AGA GCC CGG ATG C	238
		DNAJB9 R		CGC TTC TTG GAT CCA GTG	
Heat shock 70kDa protein 1B	HSPA1B	HSPA1B F	NM_005346	CGA CCT GAA CAA GAG CAT C	213
		HSPA1B R		AAG ATC TGC GTC TGC TTG G	
Insulin-induced gene 1	INSIG1	INSIG1 F	NM_198336	TAC GCT GAT CAC GCA GTT TC	239
		INSIG1 R		TCA CTA TGG GGC TTT TCA GG	
Homocysteine-inducible, ER stress inducible, ubiquitin-like domain	HERPUD1	HERPUD1 F	NM_001010990	GAG CCT GCT GGT TCT AAT CG	194
		HERPUD1 R		GAA AGC TGA AGC CAC CCA TAG	
Mitogen activated protein kinase 10	MAPK10	MAPK10 F	NM_138981	TGA AGA AAT TGC AAC CCA CA	238
		MAPK10 R		GCT GGG TCA TAC CAG ACG TTG	
ER to nucleus signaling 1	ERN1	ERN1 F	NM_001433	CGG CCT TTG CAG ATA GTC TC	226
		ERN1 R		ACG TCC CCA GAT TCA CTG TC	
ER degradation enhancer, mannosidase alpha-like	EDEM1	EDEM1 F	NM_14674	TGG ACT GCA GGT GCT GAT AG	195
		EDEM1 R		GGA TTC TTG GTT GCC TGG TAG	
CCAAT/enhancer binding protein beta	CEBPB	CEBPB F	NM_005194	CTC GCA GGT CAA GAG CAA G	192
		CEBPB R		AGC TGC TCC ACC TTC TTC TG	

Oligonucleotide primers used and their nucleotide sequences are listed in Table S1. All qRT-PCR reactions satisfied MIQE guidelines (Bustin et al. 2009): Disassociation profiles in reactions that yielded products contained single homogeneous peaks. In all reactions GAPDH was used as a normalizer. In previous qRT-PCR arrays comparing Zhangfei expressing and non-expressing cells five house keeping genes were analyzed. The levels of GAPDH were not affected by Zhangfei expression.

3.3.7 Co-immunoprecipitation

Vero cells in 6-well dishes were transfected using Lipofectamine 2000 (Invitrogen, 11668-019) with plasmids expressing Xbp1s alone or in combination with a plasmid expressing Zhangfei, tagged at its amino terminus with a FLAG epitope. Twenty-four hr after transfection MG132 (5 μM) was added. After an additional 24 hr cells were washed with PBS and lysed in 250 μl/well cold lysis buffer (50 mM Tris, pH7.5, 150 mM NaCl, 1 mM EDTA and 0.1% TritonX-100) containing protease inhibitor cocktail (Sigma, P8340). After centrifugation at 13,000 ×g at 4^{o}C mouse anti-FLAG antibody (5 μl) was added to the supernatant and the sample incubated for 12 hr with constant gentle agitation. Protein A/G agarose beads (100 μl, Fisher Scientific, 20421) was added and the samples were incubated for an additional 4 hr at 4^{o}C. Agarose beads were collected by centrifugation at 13,000 ×g and washed 4 times in lysis buffer before boiling in SDS-PAGE sample buffer. Proteins in samples of the unfractionated cell lysate or immunoprecipitates were separated by SDS-PAGE, transferred to membranes and probed with either rabbit anti-Xbp1 (Abcam, ab37152) or anti-ZF antisera. Antibodies were visualized after incubation with Alexa488-labelled anti-rabbit antibody (Invitrogen, A-11001).

3.3.8 Adult DRG culture

The study was carried out in strict accordance with the Canadian Council on Animal Care and the University of Saskatchewan animal care committee guidelines. The protocol (Role of neurotropic molecules in intact and injured neurons, protocol #19920164) was approved by the University of Saskatchewan Committee on Animals Care (UCACS) and the Animal Research Ethics Board (AREB). Dorsal root ganglia (DRG) were removed from adult male Wistar rats, treated with 0.25% collagenase (Sigma, C-0130) for 1 h at 37°C and then dissociated with 2.5 % trypsin (Sigma) for 30 min at 37°C before being plated on laminin (1 mg/mL, BD Biosciences, 354232) and poly-D-lysine-coated (25 mg/mL, Sigma) coverslips at 10^4 cells per well in a 6-well plate (BD biosciences) in DMEM (Sigma Life Science) supplemented with 10 ng/ml of NGF (Cedarlane, CLMNET-005.1). Cytosine β-D-arabinofuranoside (Ara-C, 10 mM; Sigma, C-6880) was

included to inhibit proliferation of non-neuronal cells. Rat neurons were transfected using Lipofectamine RNAiMAX (Invitrogen, 13778-150) with plasmids expressing either siRNA against Zhangfei or control siRNA. In previous experiments we had shown that this siRNA reduces Zhangfei protein levels by almost 50% and abrogates the ability of the protein to suppress the transcription factor Luman/CREB3 (Valderrama et al. 2008). Twenty four hr after transfection, cells were treated with 100 nM thapsigargin (Sigma, T-9033) for 4 hr. Cells were then harvested, RNA purified and UPR-related transcripts as well as ZF transcripts assessed by qRT-PCR as described above. To assess the efficiency with which primary neurons could be transfected with the procedure used, DRG neurons were transfected at a final concentration of 10 nM with the TYE 563 DS Transfection Control duplex (Integrated DNA Technologies) using Lipofectamine RNAiMAX Reagent (Life Technologies) according to the manufacturer's instructions. Cells were imaged 24 hours post transfection.

3.4 Results

3.4.1 Does the ectopic expression of Zhangfei influence the UPR?

We used qRT-PCR arrays designed to assess genes related to the UPR to determine if the expression of Zhangfei in cells influenced the response. Since we have shown that Zhangfei has a profound influence when ectopically expressed in ONS-76 human medulloblastoma cells (Valderrama et al. 2008; Valderrama et al. 2009), we compared RNA purified from ONS-76 cells infected with adenoviruses expressing either Zhangfei (AdZF) or the control protein β-galactosidase (AdLZ, Fig 3.1A). Except for two transcripts that were present in a two–fold excess in Zhangfei-expressing cells, we found no significant differences between the two samples. Since cells in the laboratory normally grow in unstressed conditions, we then determined if the calcium ionophore thapsigargin would induce UPR genes. Cells were treated with 100 nM thapsigargin or with an equivalent amount of DMSO, the diluent for thapsigargin, for 4 hr. The drug was then removed and cells infected with AdLZ. Forty eight hr later RNA was purified, converted to cDNA and analyzed with UPR qRT-PCR arrays (Fig 3.1B). In thapsigargin-treated cells transcripts for several UPR-related genes were significantly increased. We next compared thapsigargin-treated cells infected with either AdZF or AdLZ. Figure 3.1C shows that many UPR-related gene transcripts were reduced in Zhangfei-expressing cells. To confirm these results we independently designed primers to amplify portions of the mRNAs for the genes identified by the arrays. Results using these primers confirmed the results of the arrays (Fig 3.1D).

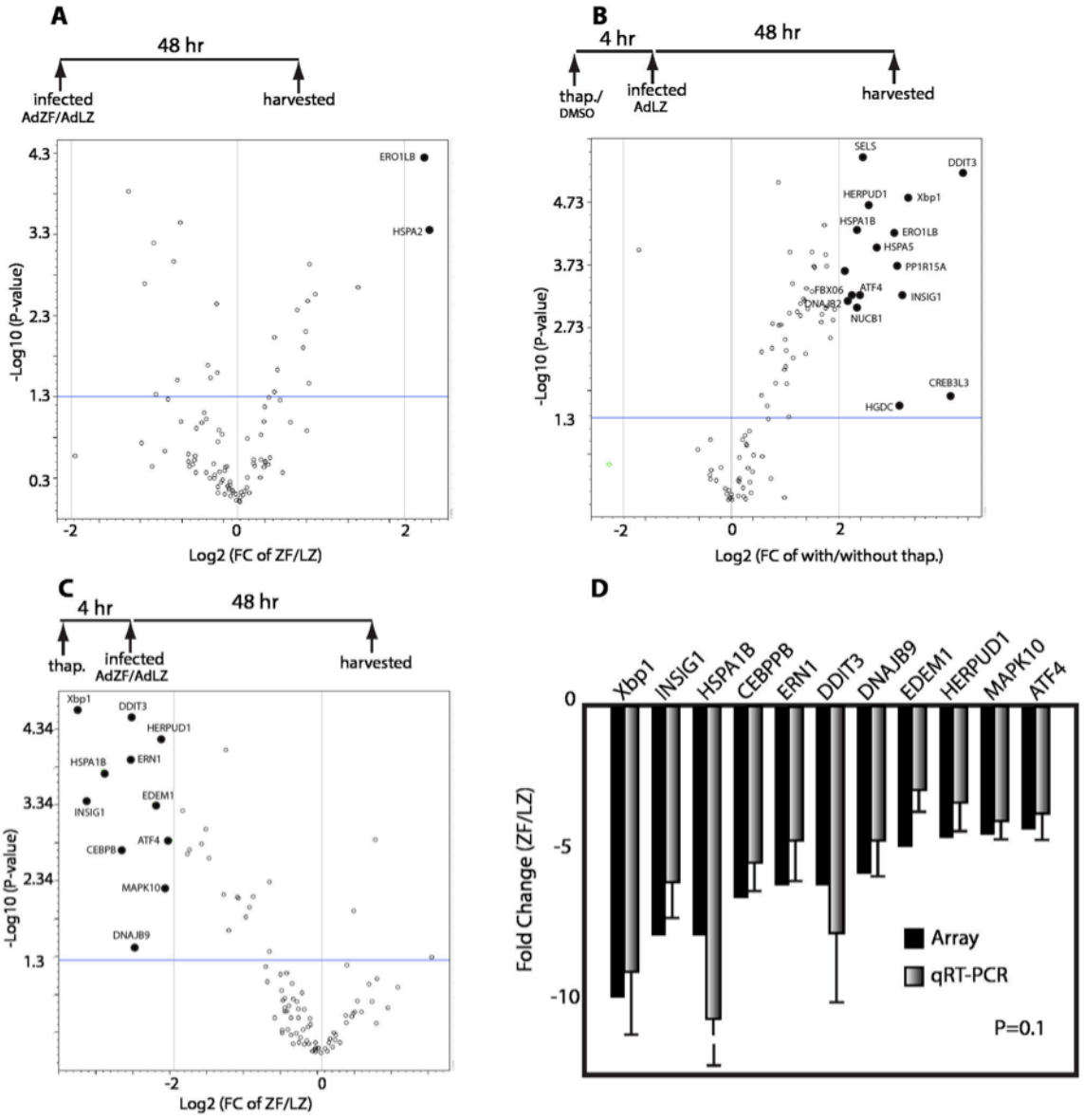

Figure 3.1 Suppression of UPR genes by Zhangfei in ONS-76 medulloblastoma cells treated with thapsigargin. A,B,C – "Volcano" plots from data derived from qRT-PCR arrays designed to monitor transcripts of genes associated with the UPR. The horizontal axes in these graphs represent log2 fold differences between the samples indicated. The vertical axis represents –log10 of *P* values for data derived from three pairs of arrays. The horizontal line (-1.3) represents a *P* value of 0.05. Transcripts that changed more than 2 fold (<-2 or >2, vertical lines) and had a *P* value <0.05 were considered significant and

are indicated by solid spots with gene designations. (A) Difference in UPR transcripts between ONS-76 cells expressing either Zhangfei or LacZ. Cells were infected with adenoviruses expressing either Zhangfei or LacZ and harvested 48 hr later for RNA extraction and analysis. (B) Differences between LacZ-expressing cells treated with either thapsigargin or DMSO (solvent for thapsigargin). Cells were treated for 4 hr then infected with adenovirus vector expressing LacZ and harvested for RNA extraction and analysis 48 hr later. (C) Effect of Zhangfei on UPR genes activated by thapsigargin. Cells were treated with DMSO or thapsigargin for 4 hr then infected with adenovirus vectors expressing either Zhangfei or LacZ and harvested 48 hr later for RNA extraction and analysis. (D) A comparison of data from the qRT-PCR arrays (C) with data from qRT-PCR experiments using primers designed "in-house". ATF4 – activation transcription factor 4CEBPB – CCAAT enhancer binding protein-beta, DDIT3 – DNA damage inducible transcript -3, DNAJB9 – homologue of DNAJ/ 40 kD heat shock protein, EDEM – ER degradation enhancer mannosidase alpha-like 1, ERN1 – ER to nucleus signaling, HERPUD1 – homocysteine-inducible ER stress inducible ubiquitin-like domain member 1, HSPA1B – heat shock 70 kD protein 1B, INSIG1 – insulin-induced gene 1, MAPK10 – mitogen-activated protein kinase 10, Xbp1 – X box binding protein 1.

To determine whether a decrease in the transcripts for UPR genes was reflected in a decrease in proteins as well, we simultaneously detected in both ONS76 and Vero cells, protein (Fig 3.2A) and RNA (Fig 3.2B) for HERP and GRP78, two components of the UPR. Cells were mock-infected (MI) or infected with an adenovirus vector expressing Zhangfei. We examined cells that expressed Zhangfei for 24 hr and then treated with thapsigargin for 4 hours (lanes 1, 2, 5 and 6). This would determine the ability of cells expressing Zhangfei to respond to the UPR. As an alternative, we treated cells with thapsigargin for 4 hr and then infected or mock infected cells (lanes 3, 4, 7 and 8). This would assess the ability of Zhangfei to turn off the UPR once it had been activated. In both experiments, mock-infected cells were an indication of the level of HERP and GRP78 without Zhangfei at the time of cell harvest. Cells already expressing Zhangfei (compare lane 1 with 2, and 5 with 6 in Fig 3.2A) were unable to make detectable amounts of HERP as compared to mock-infected cells. After 4 hr of thapsigargin treatment mock-infected cells made relatively little GRP78 (barely visible as a faint band in lanes 2 and 6) and even this was not detected in Zhangfei-expressing cells (lanes 1 and 5). When cells were treated with thapsigargin before infection or mock-infection, and then maintained in thapsigargin-free medium for 24 hr, substantial amounts of GRP78 was detected in mock-infected cells (lanes 4 and 8) and this was considerably reduced in Zhangfei–expressing cells (lanes 3 and 7—the decrease for Vero cells was less than for ONS76 cells). Zhangfei also reduced HERP as compared to mock-infected cells (compare faint band in lane 4 with lane 3). Figure 3.2B shows that in this experiment Zhangfei, before or after thapsigargin treatment, reduced levels of HERP and GRP78 transcripts. To confirm the effects of Zhangfei on GRP78 we transfected ONS76 and Vero cells with a plasmid expressing Zhangfei and then visualized ZF and GRP78 by immunofluorescence (Fig 3.2C). Both cell lines cells expressing Zhangfei lacked detectable GRP78.

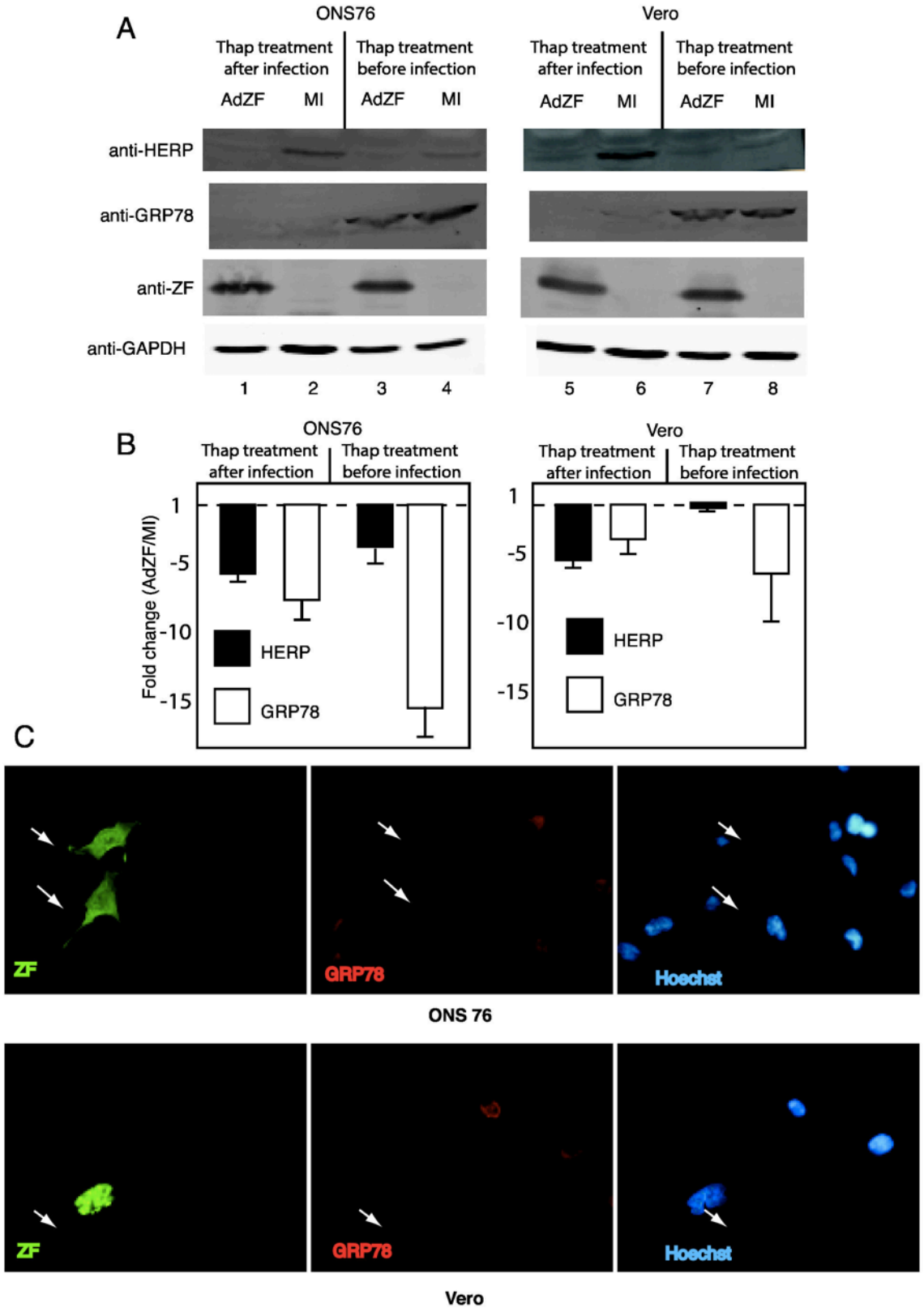

Figure 3.2 Effect of Zhangfei on HERP and GRP78 transcripts and proteins in cells

treated with thapsigargin. ONS76 and Vero cells were treated for 4 hr with thapsigargin either after infection, or mock infection, with an adenovirus vector expressing ZF (lanes 1,2, 5 and 6) or before infection (lanes 3,4, 7 and 8). Cells were harvested 24 hr after infection. Proteins in samples were separated by SDS-PAGE and probed with antibodies against HERP, GRP78 or Zhangfei (ZF) and GAPDH (A) and RNA was extracted from parallel duplicate cultures and assayed for HERP and GRP78 transcripts by qRT-PCR (B). (C) ONS-76 and Vero cells were transfected with plasmids expressing FLAG epitope linked to the coding sequences of Zhangfei. Cells were fixed and incubated with a mixture of mouse monoclonal antibody against FLAG and rabbit antibody against GRP78 followed by Alexa 488 linked anti-mouse and Alexa 546-linked anti-rabbit antibodies. Cells were also stained with Hoechst to stain nuclei. Arrows identify Zhangfei (ZF) expressing cells.

3.4.2 Can Zhangfei suppress the ability of Xbp1s to activate transcription and is its leucine-zipper required?

Since the published literature suggests that at least in some cells Zhangfei is relatively ineffective against ATF6 (Misra et al. 2012), we examined the effect of Zhangfei on the UPR-regulating transcription factor Xbp1s. In transiently transfected cells Xbp1s can activate transcription of reporter genes linked to unfolded protein response elements (UPRE). To determine if Zhangfei could suppress this ability of Xbp1s, Vero cells were transfected with a CAT-reporter plasmid linked to multiple copies of UPRE (Fig 3.3 A and B). Cells were also transfected with plasmids expressing the spliced form of Xbp1, Xbp1s, and increasing amounts of a plasmid expressing Zhangfei (Fig 3.3A). Zhangfei inhibited the activity of Xbp1s in a dose-dependent manner (solid circles). The immunoblot in Figure 3.3D shows that increasing the amount of the Zhangfei-expressing plasmid in the transfection mixtures also led to increased amounts of the protein in the cells. To determine if the leucine-zipper of Zhangfei was required for its suppressive activity, parallel cultures in the experiments described above were transfected with a plasmid expressing a mutant of Zhangfei in which the first 6 consecutive leucine residues in the bLZip domain were replaced with alanine residues (ZF(L/A)). Unlike Zhangfei, ZF(L/A) did not suppress Xbp1s (Fig 3.3, A,B). Figure 3.3D shows that the mutations in ZF(L/A) did not affect its stability. Interestingly, not only did ZF(L/A) not suppress Xbp1s, it consistently appeared to enhance the activities of Xbp1s almost 2 fold (Fig 3.3A and B). This enhancement did not reflect an increased ability of ZF(L/A) to activate transcription as neither Zhangfei nor ZF(L/A) on their own, had any effect on a UPRE containing promoter (Fig 3.3B).

To confirm that Zhangfei required its leucine zipper to interact with Xbp1s, we conducted two-hybrid assays in which activation of a reporter gene depended on interaction of Xbp1s with Zhangfei or ZF(L/A) tethered to a promoter by the DNA-binding domain of the yeast transcription factor Gal4 (UAS). Cells were transfected with a reporter plasmid with a Gal4 UAS containing promoter and either Gal-ZF or Gal-ZF(L/A) with or without plasmids expressing Xbp1s. The results in Figure 3C show that the reporter activity in the presence of Gal-ZF was significantly greater than the activity in the presence of Gal-

ZF(L/A).

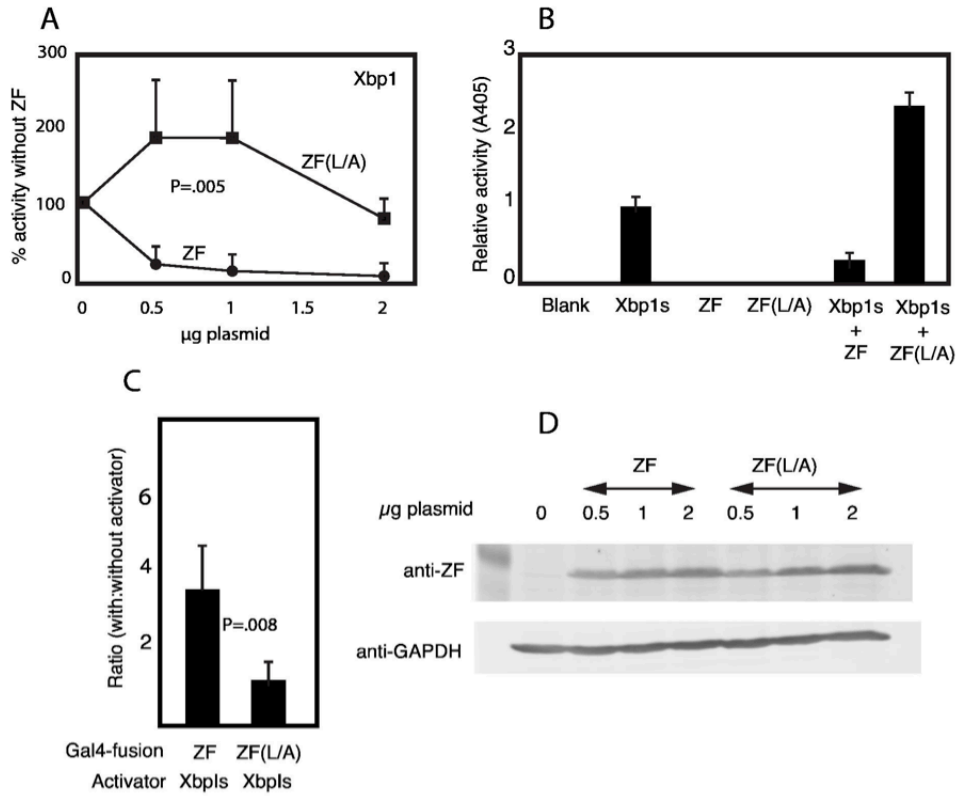

Figure 3.3 Zhangfei suppresses the ability of Xbp1s to activate transcription and requires its leucine zipper to do so. (A) and (B). Vero cells were transfected with a plasmid containing the coding sequence for CAT linked to a promoter with three copies of the unfolded protein response element as well as a plasmid expressing Xbp1s and varying amounts of plasmids expressing either Zhangfei (ZF) or a mutant, ZF(L/A) in which all leucine residues in the LZip domain had been replaced with alanines. All samples also contained, as a control, a plasmid expressing b-galactosidase. The CAT activity in each sample was normalized to this internal control and expressed as a percentage of the activity in samples containing no vector expressing either ZF or ZF (L/A). The total amount of DNA in each transfection was made up to 5 µg with "empty" expression vector (pcDNA3). Bars indicate standard deviation from the mean. (B) ZF(L/A) does not activate a promoter containing UPRE but enhances the activity of Xbp1s. (C) ZF interacts with Xbp1 with its leucine zipper. Cells were transfected with a vector with the coding sequence for CAT linked to three copies of a sequence, UAS, that

binds the DNA-binding domain of the yeast protein GAL4. Cells also received plasmids expressing either ZF or ZF(L/A) linked to the Gal4 DNA-binding domain and either an "empty" expression vector or vectors expressing Xbp1s. Bars represent the ratio of the relative CAT activity (normalized to the internal control, b-galactosidase) of samples with Xbp1s to samples with no activator ("empty" vector). (D) An immunoblot showing that vectors with cloned ZF or ZF (L/A) express the proteins in a dose-dependent manner. The results represent the averages of three experiments assayed in duplicate. Bars in all figures represent standard deviation from the mean and *P* values are indicated on the figures.

3.4.3 How does Zhangfei suppress Xbp1?

Zhangfei may suppress Xbp1s by either causing its degradation, inhibiting its ability to bind to cognate promoters or by sequestering it from the nucleus. To test the first possibility we examined Xbp1s in cells expressing Zhangfei. Vero cells were transfected with either a plasmid expressing Xbp1s alone or with a plasmid expressing Zhangfei. In cells expressing both proteins we were unable to detect Xbp1s (Fig 3.4A, compare lane 2 with lane 3). In contrast, the co-expression of both proteins did not lead to an appreciable decrease in Zhangfei (compare lanes 1 and 3). The proteasome inhibitor, MG132 restored Xbp1s levels in Zhangfei-expressing cells (lane 6). To determine whether the leucine zipper of Zhangfei was required for the induced degradation of Xbp1s, we expressed Xbp1s with increasing amounts of plasmid expressing either Zhangfei or ZF(L/A). Figure 3.4B (Fig 3.4C shows densitometer measurements of repeated experiments) shows that at low concentrations protein (0.5 and 1 μg expression plasmid) there was an obvious difference in the ability of Zhangfei and its LZip mutant to induce the degradation of Xbp1s. At higher concentrations (2 μg of plasmid, not shown) the differences between the mutant and wild-type Zhangfei were less pronounced. Figure 3.5, which shows intracellular proteins detected by immunofluorescence, supports these data—we were unable to detect Xbp1s in cells expressing Zhangfei, while the LZip mutant, ZF(L/A), had no effect. When Xbp1s and ZF(L/A) were expressed together both proteins colocalize in the nucleus (Fig 3.5, bottom row) with higher concentrations in nuclear structures. This suggests that nuclear localization of Xbp1s does not rely on association with Zhangfei.

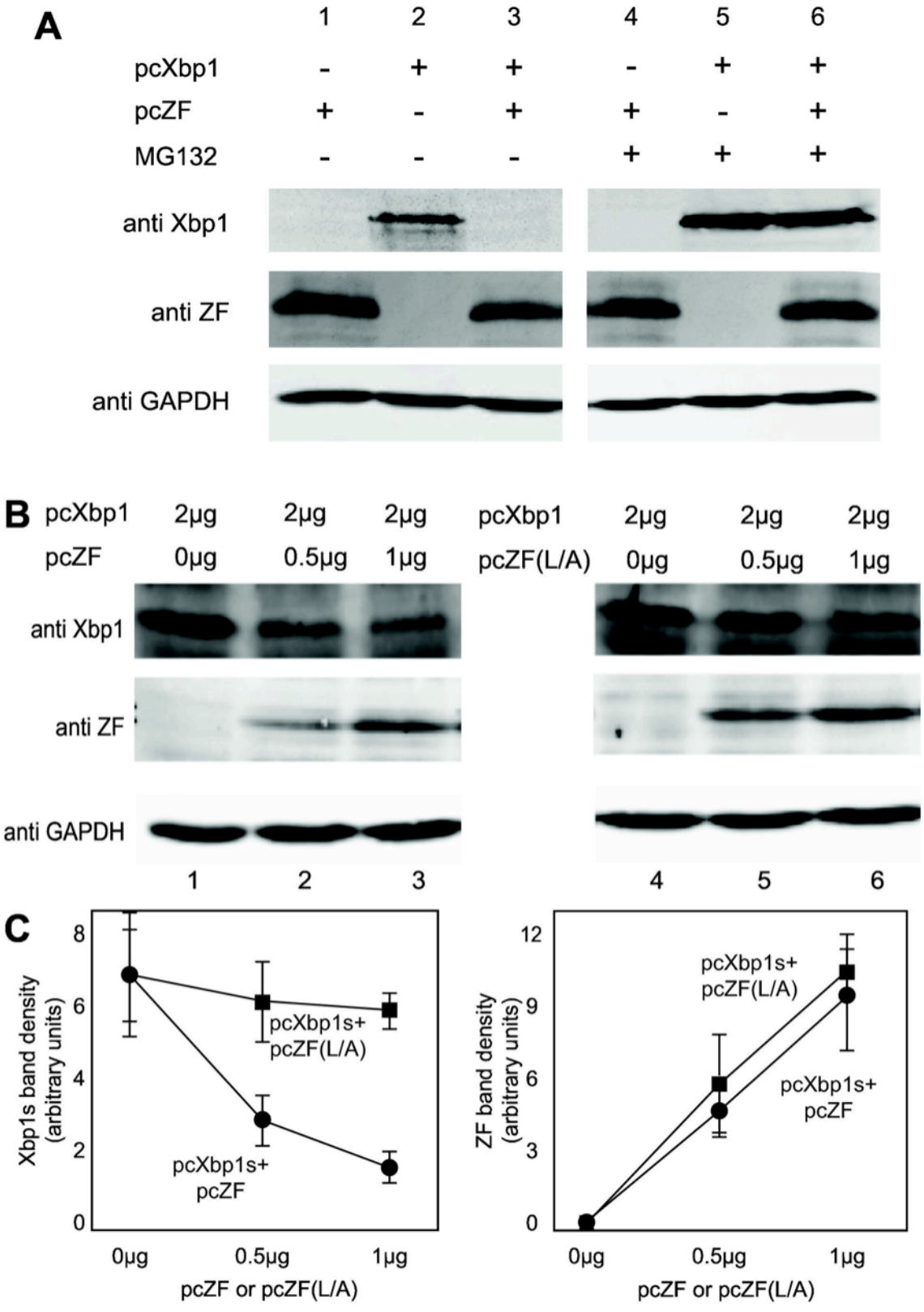

Figure 3.4 Zhangfei directs Xbp1 for proteasomal degradation and requires its

leucine zipper to do so. (A) Vero cells were transfected as indicated with plasmids expressing Xbp1s and an empty vector or plasmid expressing Zhangfei. Cells either received 5 μM MG132 or an equivalent volume of carrier, DMSO. Cells were harvested 24 hr later and proteins detected by immunoblotting. (B) Vero cells were transfected with a plasmid expressing Xbp1s alone or increasing amounts of a plasmid expressing Zhangfei or a mutant in which all leucine residues in the zipper had been changed to alanine - pcZF(L/A). Cells were harvested 24 hr later and proteins detected by immunoblotting. The density of each band on the immunoblot was estimated by densitometry and normalized to the density of the GAPDH band in the sample. Average values and standard deviation from three experiments are shown in (C).

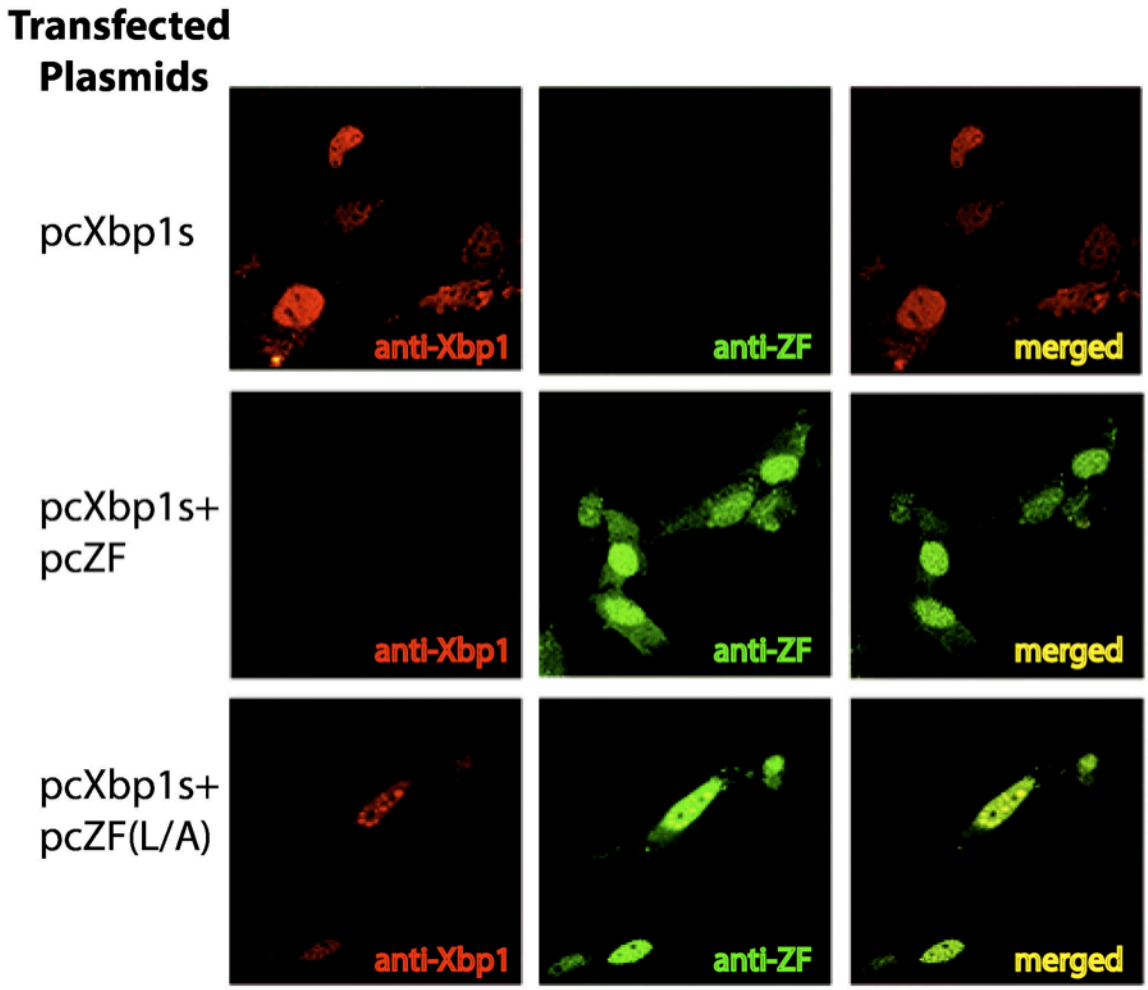

Figure 3.5 Immunofluorescent images showing the absence of Xbp1 in cells expressing Zhangfei but not Zhangfei with a mutated leucine zipper. Vero cells were transfected with plasmids expressing either Xbp1 alone or in combination with either Zhangfei or ZF(L/A). Cells were then fixed and incubated with a mixture of rabbit anti-Zhangfei and mouse anti-Xbp1 antibodies. Antibodies were visualized by staining with Alexa546 (red) anti mouse and Alexa488 (green) anti-rabbit antibodies.

3.4.4 Does Zhangfei interact with Xbp1s?

To determine if Zhangfei and Xbp1s interacted, we co-expressed the proteins in Vero cells. Zhangfei coding sequences included a FLAG epitope. Since our previous experiments indicated that interactions between the two proteins might lead to the proteasomal degradation of Xbp1s (Fig 3.4A), we treated cells with MG132 to suppress degradation. From the lysates of these cells we precipitated Zhangfei and associated proteins with monoclonal antibodies against FLAG and then detected Xbp1 or Zhangfei in the immunoprecipitates using immunoblots antisera against either Xbp1 or Zhangfei. Figure 3.6A shows that in cells expressing both proteins they were in a stable association (lane 6). In a similar experiment, Xbp1 did not precipitate with ZF(L/A) (Fig 3.6B, compare lanes 7 and 8) confirming our results that the leucine-zipper of Zhangfei was required for the interaction.

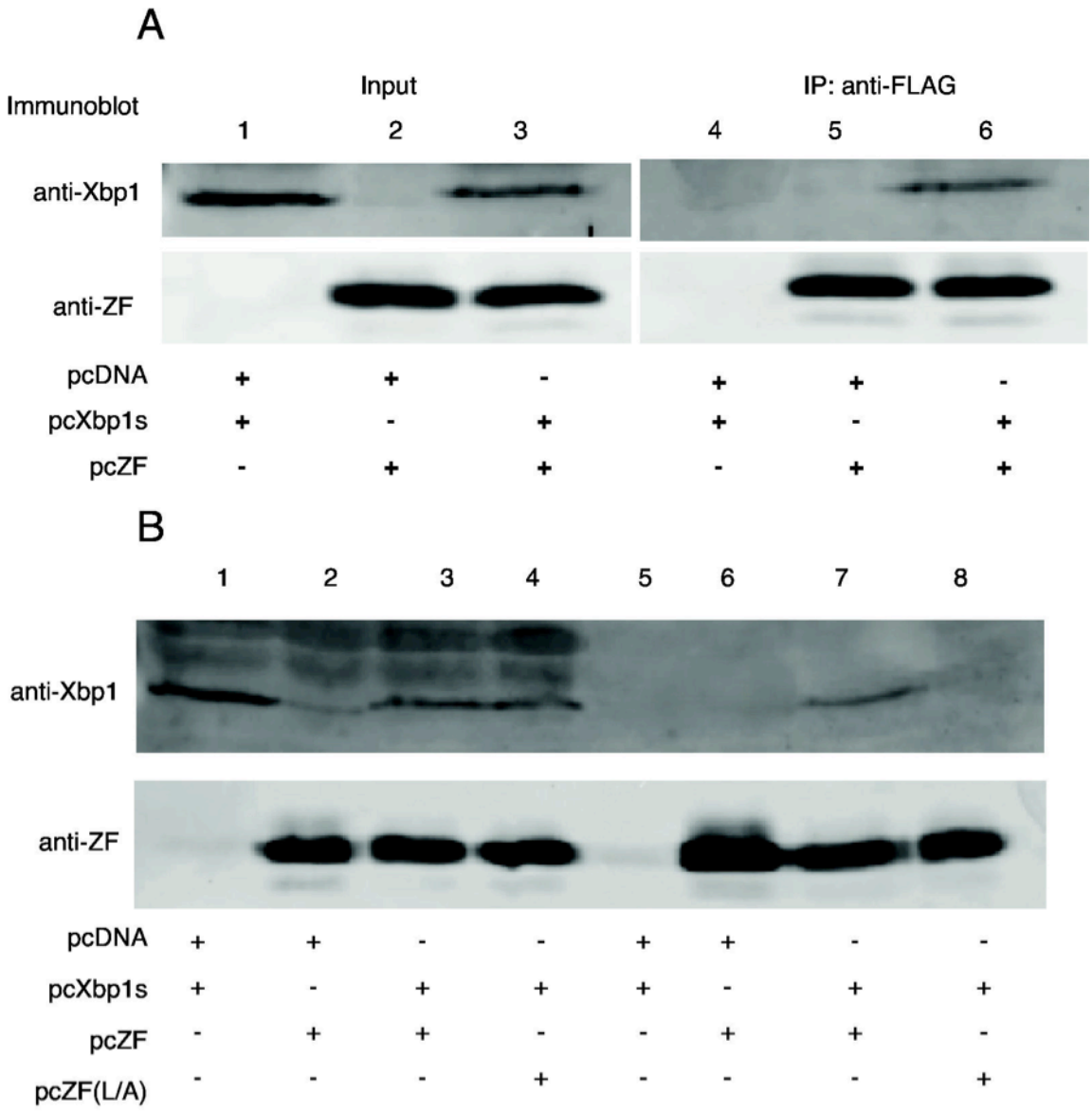

Figure 3.6 Zhangfei co-immunoprecipitates with Xbp1s in MG132-treated cells. (A) Vero cells were transfected to express Xbp1s and FLAG-tagged Zhangfei either on their own alone or together. The cells were treated with MG132 to reduce proteasomal degradation and, 24 hr later, cells lysates were immunoprecipitated with mouse anti-FLAG antibody. The precipitates were separated by SDS-PAGE and Xbp1 and Zhangfei detected in immunoblots with rabbit anti-Xbp1 or anti-Zhangfei antisera. Lanes 1-3, represent cell lysates without immunoprecipitation while lanes 4-6 are immunoblots of material precipitated by anti-FLAG antibodies. (B) A similar experiment as in A, showing that Xbp1s was not co-precipitated with the Zhangfei mutant ZF(L/A).

3.4.5 Can endogenous Zhangfei suppress the UPR in sensory neurons?

We had previously detected Zhangfei in mature neurons of the central nervous system and in sensory neurons in trigeminal ganglia (Akhova et al. 2005). To determine if endogenous Zhangfei could suppress the UPR in these cells, disassociated neurons from adult rat dorsal root ganglia were transfected with plasmids expressing either siRNA against Zhangfei or control siRNA (Valderrama et al. 2008). The UPR was then induced in these cells with thapsigargin. Levels of transcripts for Xbp1s, unspliced Xbp1 (Xbp1us), CHOP, GRP78 and Zhangfei as well as protein levels for Xbp1s, GRP78, HERP, GAPDH and Zhangfei were then measured using qRT-PCR, and immunoblotting and densitometry. The primers used for qRT-PCR were directed against conserved regions of the coding sequences of these genes. The results (Fig 3.7A and 3.7B) show that in cells in which siRNA against Zhangfei had reduced its endogenous transcripts and protein (Fig 3.7B and 3.7C), transcripts and proteins for several UPR-related genes were increased. There was a trend towards an increase in Xbp1us transcripts although the difference from control siRNA-expressing cells was not significant. These results suggested that endogenous Zhangfei has the capacity to modulate the UPR. To demonstrate that most cells in the primary sensory neuron culture could be transfected we transfected the cells with fluorescent double stranded RNA. Figure 3.7D shows that most cells in the culture were capable of taking up transfected DNA.

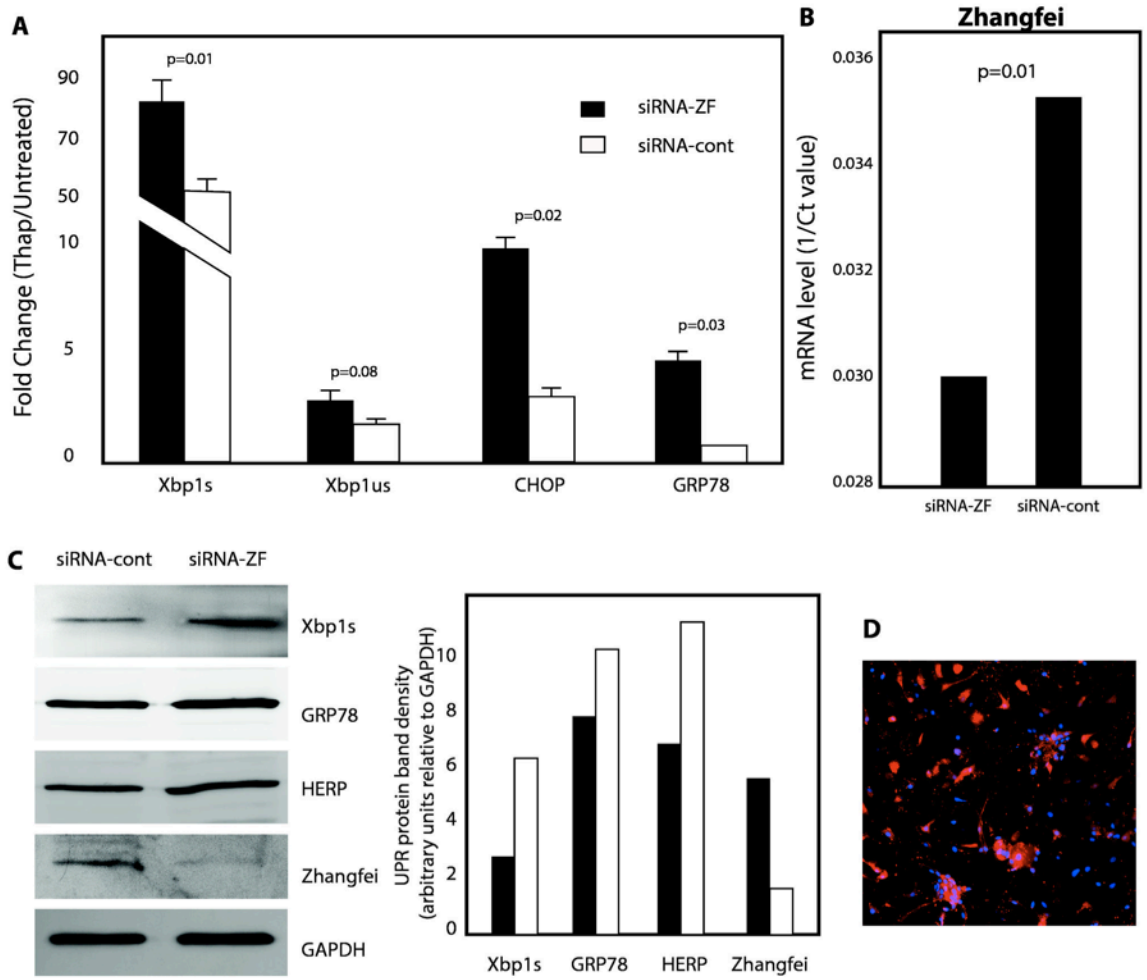

Figure 3.7 Endogenous Zhangfei suppresses the activation of UPR genes in rat peripheral neurons. Primary rat dorsal root neurons were transfected with plasmids expressing either control siRNA or siRNA against Zhangfei (siRNA-ZF). The next day cells were treated with DMSO or thapsigargin and 4 hr later RNA was harvested for qRT-PCR analysis using primers designed to amplify Xbp1s, CHOP, GRP78, Xbp1us (A) or Zhangfei (B). (A) Fold increases in RNA between DMSO and thapsigargin treated samples comparing siRNA-ZF (black bars) and siRNA control (white bars) expressing cells. (B) Effect of si-ZF on transcripts levels of endogenous Zhangfei. Results are expressed as 1/Ct. Columns represent averages of triplicate samples with bars as standard deviation from the mean. (C) Suppression of endogenous Zhangfei increases Xbp1s, Grp78 and HERP proteins. Lysates of neurons transfected with either siRNA-ZF or siRNA-control were analyzed by immunoblotting using antibodies against UPR-related

proteins. Bands on immunoblots of the left were scanned and band densities relative to the internal standard GAPDH are on the graph on the right. (D) Transfection efficiency test of siRNA. Primary rat dorsal root neurons were transfected at a final concentration of 10 nM with the TYE 563 DS Transfection Control duplex, and were imaged 24 hours post transfection (Red: marked siRNA; blue: nuclear).

3.5 Discussion

The inability of the UPR to restore normal ER function results in apoptosis. However, a successful homeostatic response should result in modulation of the UPR and resumption of normal cellular function. To do this several UPR induced proteins such as p58IPK (Rutkowski et al. 2007), NCK1 (Latreille and Larose 2006; Cardin et al. 2007) and GADD34 (Ma and Hendershot 2003; Zhou et al. 2011) feed-back to relieve the inhibition of protein synthesis mediated by PERK. These act by recruiting phosphatases that dephosphorylate eIF2 a. In addition, the product of unspliced Xbp1 mRNA binds Xbp1s and ATF6 through bLZip interactions and targets the proteins for proteasomal destruction (Yoshida et al. 2006; Yoshida et al. 2009).

Our qRT-PCR array data revealed a global significant decrease in transcripts for genes activated by the UPR inducer thapsigargin in ONS-76 human medulloblastoma cells when Zhangfei was expressed in these cells (Fig 3.1). In contrast, Zhangfei had no effect on the UPR gene transcripts in cells growing normally under favourable non-stressful conditions. This suggests that Zhangfei did not globally suppress transcription in cells but directly or indirectly targeted the expression of certain genes. Mature neurons are one of the few cell types in which we have been able to detect endogenous Zhangfei (Akhova et al. 2005). In support of our data, we also showed that selective suppression of Zhangfei by siRNA in adult primary sensory neurons from rat dorsal root ganglia increased levels of transcripts and proteins for UPR-related genes (Fig 3.7) confirming the ability of the protein to suppress the UPR once it had been activated, by thapsigargin in this case.

To determine how Zhangfei might modulate the UPR, we focused on the ability of Zhangfei to suppress the ability of Xbp1s, one of the main bLZip mediators of the UPR. We found that in cells transfected to transiently express Xbp1s as well as Zhangfei, the ability of Xbp1s to activate transcription was suppressed (Fig 3.3). This was confirmed by a corresponding decrease in the level of the corresponding UPR proteins (Fig 3.2). While this can be explained, at least to some extent, by the effect of Zhangfei on the ability of Xbp1s to initiate transcription of UPR genes, we are puzzled how Zhangfei suppressed levels of the spliced Xbp1 transcripts themselves. The bLZip transcription

factor responsible for Xbp1 transcription has not been unambiguously identified however our earlier data (Bergeron et al. 2013) as well as that of others show that activation of the UPR leads to an increase in spliced Xbp1 mRNA without a corresponding increase in unspliced transcripts. In addition Figure 3.4 shows that while suppression of Zhangfei by siRNA decreased levels of spliced Xbp1 mRNA it did not have a significant effect on unspliced transcripts. This suggests that Zhangfei may have an effect on the IRE1-mediated cytoplasmic splicing process in addition to suppressing transcription by Xbp1s.

Basic leucine-zipper motif containing proteins are known to interact with other bLZip proteins through their leucine zippers and can be categorized according to whether they strongly favour the formation of homo-dimers, hetero-dimers or both (Vinson et al. 2002). In addition, Newman and Keating (Newman and Keating 2003) using peptide arrays and fluorescent bLZip probes, measured interactions between the bLZip regions of all human and yeast bLZip proteins. According to their results the Xbp1 and ATF6 domains were the only ones among the 49 bLZip proteins tested that showed a strong association with the Zhangfei domain. Binding between Zhangfei and ATF4 could only be detected when the fluorescent Zhangfei peptide was used to detect binding to the array.

Since bLZip proteins can act as co-activators by interacting with other transcription factors by mechanisms that do not rely on their bLZip domains (Wardell et al. 2002), we determined whether the suppressive effect of Zhangfei on Xbp1s required its leucine zipper. Our data strongly suggest that it does (Figs 3.3, 3.4, 3.5 and 3.6). A mutant of Zhangfei in which all 6 consecutive leucines in the zipper were replaced with alanine was less efficient at suppressing Xbp1s than Zhangfei with an intact zipper. Further, our results in the *in vivo* protein hybrid assay in which transcriptional activation relied upon interaction of Gal4-linked Zhangfei or its mutant with the Xbp1s (Fig 3.3C), as well as our inability to co-precipitate Zhangfei (L/A) and Xbp1s, support our observations.

Interestingly, not only did Zhangfei (L/A) not suppress Xbp1s, it consistently enhanced its ability to activate transcription (Fig 3.3 A and B). A possible explanation for this phenomenon may lie in the co-localization of the two proteins in nuclear domains (Fig 3.5, bottom row). We have observed that if proteasomal degradation is suppressed,

Zhangfei co-localizes with Luman/CREB3 (another bLZip protein that it suppresses) in intranuclear promyelocytic leukemia protein-containing nuclear domains (Misra et al. 2005). These domains are sites for nuclear proteasomes (St-Germain et al. 2008) and it is possible that, on their own, both Zhangfei and Xbp1s are normally targeted to these sites. Expressed together, Zhangfei enhances the degradation of Xbp1s while Zhangfei (L/A), unable to bind Xbp1s, has a suppressive effect on proteasomes.

The interaction between Zhangfei and Xbp1s results in the proteasomal degradation of Xbp1s (Fig 3.4). Elucidating the mechanism by which Zhangfei targets Xbp1s for proteasomal degradation will require additional work. However, several bLZip proteins have been shown to target other proteins for such destruction. Thus, the human T-cell leukemia virus coded bLZip protein HBZ and the host transcription factor maculoaponeurotic fibroma (Maf) homologue B interact through their bLZip domains following which MafB is targeted for proteasomal degradation (Mukai and Ohshima 2011). HBZ also interacts with host interferon response factor 1 (IRF1) and targets it for degradation (Ohshima et al. 2010). Some interactions between bLZip proteins, such as between ATF5 and nucleoplasmin (Liu et al. 2012) lead to the ubiquitination of the target prior to degradation suggesting a mechanism for proteasomal targeting. Recently a SUMO-conjugase, UBC9, was shown to stabilize Xbp1s by interacting with its bLZip domain. Displacement of UBC9 from Xbp1s by Xbp1u led to decrease in the stability of Xbp1s (Liu et al. 2012). Zhangfei may, in a similar manner, destabilize Xbp1s by displacing UBC9 from its bLZip domain. SUMOylation of proteins prevents ubiquitination (Johnson 2004) and subsequent proteasomal degradation. Interestingly, UBC9 did not require its SUMOylating activity to stabilize Xbp1s (Liu et al. 2012) suggesting that UBC9 exerts its effect by other means.

While we have shown a direct effect of Zhangfei on Xbp1s, Zhangfei may suppress the other UPR-inducing bLZip factors as well. Although ATF6 is one of the best-characterized of these factors, other ER-resident bLZip proteins such as Luman/CREB3 and CREBH are thought to perform this role in some cell types (reviewed in (Asada et al. 2011; Chan et al. 2011)). We have previously shown that Zhangfei can suppress the activity of Luman/CREB3 but not ATF6. Recently, Misra and others (Misra et al. 2012)

showed that in hepatoma cells Zhangfei/SMILE suppresses the ability of CREBH to induce UPR genes. While further studies about the role of Zhangfei in regulation the UPR are clearly needed, our results and those of others show that Zhangfei has the ability to suppress Xbp1 as well as other bLZip proteins that may substitute for the ATF6-arm of the ER-stress sensing pathways. However, since we have only been able to detect Zhangfei in mature, differentiated neurons, its influence is likely restricted to a few cell types. Alternatively, given its dramatic effect on cell division (Valderrama et al. 2009; Bergeron et al. 2013), it may be expressed in a wider array of cells but only in a very transient manner, when it is needed.

3.6 Acknowledgements

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4. Effects of Cyclic AMP Response Element Binding Protein – Zhangfei (CREBZF) on the Unfolded Protein Response and cell growth are exerted through the tumour suppressor p53

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This Chapter demonstrated the mechanisms by which Zhangfei suppressed the UPR and cell growth in some osteosarcoma cell lines, but not in normal cells and other cancer cell lines, based on the hypothesis that *Zhangfei mediates its effect on cell growth and the UPR through an intermediary, p53, that is either not induced or is defective in cells that it does not affect*. The manuscript has been published as "Effects of Cyclic AMP Response Element Binding Protein – Zhangfei (CREBZF) on the Unfolded Protein Response and cell growth are exerted through the tumour suppressor p53" in *Cell Cycle.* 13: 279-292. by Zhang, R. and Misra, V. and is reproduced here with the permission of the copyright owner.

My contributions to this manuscript include: I designed and performed all the experiments mentioned in this manuscript. I generated the first complete draft of the submitted manuscript. Professor Vikram Misra and I worked together to revise this draft prior to submission. We also worked together on the revision.

4.1 Abstract

Zhangfei/CREBZF, a basic region-leucine zipper (bLZip) transcription factor, is a potent suppressor of growth and the Unfolded Protein Response (UPR) in some cancer cell lines, including the canine osteosarcoma cell line, D-17. However, the effects of Zhangfei are not universal and it has no obvious effects on untransformed cells and some cancer cell lines (Chapter 2), suggesting that Zhangfei may act through an intermediary that is either not induced or is defective in cells that it does not affect. Here we identify the tumour suppressor protein p53 as this intermediary. We show the following: In cells ectopically expressing Zhangfei the protein stabilizes p53 and co-localizes with it in cellular nuclei, the bLZip domain of Zhangfei is required for its profound effects on cell growth and interaction with p53. Suppression of p53 by siRNA at least partially inhibits the effects of Zhangfei on the UPR and cell growth. The effects of Zhangfei on D-17 cells is mirrored by its effects on the p53-expressing human osteosarcoma cell line U2OS while Zhangfei has no effect on the p53-null osteosarcoma cell line MG63. In U2OS cells Zhangfei displaces the E3 ubiquitin ligase mouse double minute homologue 2 (mdm2) from its association with p53 suggesting a mechanism for the effects of Zhangfei on p53.

Key words: cell cycle, protein domains, p53, osteosarcoma, protein translocation, Zhangfei/CREBZF, unfolded protein response, mdm2, basic-leucine zipper domain

4.2 Introduction

Zhangfei/CREBZF/SMILE was first discovered as a binding partner for Host Cell Factor (HCF), a co-activator of the herpes simplex virion transcription factor VP16 (Lu and Misra 2000b). The primary structure of the protein contains a leucine zipper, a basic region that lacks an asparagine residue conserved in most bLZip proteins, three potential nuclear factor binding domains (LLXXLL, where L is a leucine residue and X is any amino acid), and a domain for binding HCF. Zhangfei interacts with several proteins, possibly through its nuclear receptor and HCF binding domains as well as its leucine zipper. While Zhangfei can activate gene expression through factors such as p53 (Lopez-Mateo et al. 2012) and ATF4 (Hogan et al. 2006), it suppresses the activity of a number of transcription factors which include nuclear receptors (Xie et al. 2008; Xie et al. 2009a; Xie et al. 2009b), bLZip containing proteins such as CREBH (Misra et al. 2012), Luman/CREB3 (Misra et al. 2005), Xbp1 (Zhang et al. 2013) and SMAD 1,5,8 (Lee et al. 2012a) and the HCF-binding VP16 (Akhova et al. 2005). In the previous studies (Chapter 3), we also showed that Zhangfei had a profoundly suppressive effect on the Unfolded Protein Response (UPR) at least partly because it targets Xbp1s, an important UPR mediator, for proteasomal degradation (Zhang et al. 2013).

We have detected Zhangfei protein in differentiated neurons, but not in developing neurons or cells of neuronal tumours (Akhova et al. 2005), nor in osteosarcoma cell lines. The ectopic expression of Zhangfei in several tumour cells lines derived from medulloblastomas (ONS-76, UW228) (Valderrama et al. 2009), and osteosarcomas (D-17) (Bergeron et al. 2013) causes the cells to stop growing and display markers of apoptosis (Chapter 2). However, the suppressive effect of Zhangfei on the growth of cells is not universal. While the protein has a profound effect on some cells, it has no effect on others such as MRC5 fibroblasts (Valderrama et al. 2009). This suggests that Zhangfei may act through an intermediary that is either not induced or is defective in cells that it does not affect. The objective of this study was to identify such an intermediary and to determine how Zhangfei exerted its effect. Herein, we identified that Zhangfei suppressed cell growth and the UPR in osteosarcoma cells through direct interaction with tumour suppressor protein p53. We demonstrated that Zhangfei stabilized p53 and promoted its

nuclear retention by displacing the E3 ubiquitin ligase, mdm2. Overall, our findings reveal a novel mechanism by which Zhangfei may inhibit tumour growth and metastasis. This may provide an alternative modality for the therapy of certain types of osteosarcoma, and perhaps other tumours with functional p53.

4.3 Materials and Methods

4.3.1 Cells and tissue culture

The canine osteosarcoma D-17 cells were obtained from the American Type Tissue Culture Collection, were grown in MEM-Alpha containing 10% fetal bovine serum. Human MG63 and U2OS osteosarcoma cells were obtained from Dr. Douglas H. Thamm, (Associate Professor of Oncology, Animal Cancer Center, Colorado State University) and grown in Dulbecco's minimal essential medium containing penicillin and streptomycin and 10% newborn calf serum. All media, serum and antibiotics were purchased from Invitrogen. D-17 cells were treated with doxorubicin (0.5μM, WCVM's Accredited Veterinary Pharmacy, University of Saskachewan) for p53 induction. Stock solutions of MG132/Z-L-L-L-A (10mM, Sigma, C2211) were prepared in dimethyl sulfoxide (DMSO) and used at a concentration of 5μM. Equivalent volumes of DMSO were added to untreated cultures.

4.3.2 Plasmids

The construction of pcZF (Lu and Misra 2000b), a plasmid that expresses Zhangfei in mammalian cells, has been described. The plasmids expressing Zhangfei mutant pcZF Zip(L>A), in which all leucines in the leucine-zipper domain were replaced with alanines, was constructed by subcloning a 265 bp synthetic DNA fragment (IDT) bracketed by NotI and SgrA1sites into the corresponding coding sequences of Zhangfei between unique N*ot1* and S*grA1* sites in pcZF. The plasmids expressing Zhangfei mutant pcZF Basic del, in which basic region was deleted, was constructed by site directed loop-out mutagenesis using 36 base complementary oligonucleotides that bracketed the 25 codons for the basic region of Zhangfei. The CAT reporter plasmid pCAT3B-p53RE was constructed by transferring two copies of p53 responsive element, GGTCAAGTTGGGACACGTCCaaGAGCTAAGTCCTGACATGTCT, to pCAT3Basic (Promega). Oligonucleotides representing the p53 responsive elements with overhanging 5' terminal KpnI and 3' terminal BglII sites were purchased from IDT company. The oligonucleotides were annealed and ligated pCAT3Basic cut with the same enzymes. The plasmids expressing the full length of wild-type p53 were constructed by subcloning the

HindIII – XhoI cDNA fragment containing the entire coding sequence of p53 from U2OS cells between the HindIII and XhoI sites in the multiple restriction site cloning regions of the mammalian expression vector pcDNA3 (Invitrogen, V79020). The specific primers for p53 amplification are: forward: 5'-GACACGCTTCCCTGGATTGGC-3', reverse: 5'-TCAGTCTGAGTCAGGCCCTT-3'. The veracity of all cloned wild-type and mutants' coding sequences were confirmed by sequencing

4.3.3 Transfection and CAT Assays

Cells were transfected with plasmids using Lipofectamine 2000 (Invitrogen, 11668-019) as described in manufacturer's instructions. Five μg of DNA was used for 5×10^5 cells for chloramphenicol acetyl transferase (CAT) assays. For CAT assays, 250 ng of pCMVBGal, a plasmid specifying β-galactosidase, were added to each transfection. Lysates were assayed for β-galactosidase (Sambrook and Russell 2001) and for CAT using an enzyme-linked immunosorbent assay kit (Roche Applied Science, 11-363-727-001). CAT values were adjusted for transfection efficiency using β-galactosidase values. In figures for CAT assays, each datum point is the average of duplicate transfections with the bar representing the range. The data are representative of several (at least two) independent experiments that gave the same results.

4.3.4 RNA interference

p53-specific siRNA (Invitrogen, TP53VHS40366) or Stealth RNAi™ siRNA negative control (Invitrogen, 12935-300) (1pmol/5000 cells) were transfected into cells with Lipofectamine 2000 (Invitrogen, 11668-019) as described in manufacturer's instructions. These p53-specific siRNAs are homologous to human as well as canine p53, which was confirmed by comparing the sequence of human or canine p53 with siRNA sequence.

4.3.5 Adenovirus Vectors Expressing Zhangfei (Adeno-ZF) and β-galactosidase (Adeno-LacZ)

These vectors were constructed, grown, and purified using the Adeno-X Expression System (Clontech, K1650-1) as described earlier (Misra et al. 2005). Cells were infected with adenovirus vectors expressing either Zhangfei (Adeno-ZF) or a control protein—*E.*

coli b-galactosidase, (Adeno-LacZ) or were mock-infected. A multiplicity of infection (MOI) of 100 plaque-forming units (pfu) per cell was used.

4.3.6 Antibodies, immunoblotting and immunofluorescence

The antibodies used were rabbit anti-Zhangfei serum, mouse anti-p53 (Santa Cruz Biotechnology, DO-1 sc-126), rabbit anti-p21 (Santa Cruz Biotechnology, C-19, sc-397), rabbit anti-mdm2 (Santa Cruz Biotechnology, C-18, sc-812), and mouse anti-GAPDH (Sigma, G8795-200UL). Secondary antibodies were goat anti-mouse Alexa 488 (Invitrogen, A-11001), goat anti-rabbit Alexa 546 (Invitrogen, A-11035) and goat anti-rabbit Cy5 (Invitrogen, A-10523). Cells were processed for immunoblotting and immunofluorescence as described previously (Lu and Misra 2000b; Bergeron et al. 2013). Images were captured using a digital camera attached to a Zeiss Axioskop microscope (Axiovert 135) and Northern Eclipse software (EMPIX Imaging). Captured images were processed using Adobe Photoshop and Illustrator CS6 software.

4.3.7 Quantitative real-time PCR

Total RNA was extracted using RNeasy Plus Mini Kit (Qiagen, 74136). Gene expression was analyzed by RT-PCR using Brilliant II SYBR Green QPCR Master Mix Kit (Agilent Technologies, 600828). The primers used were: primers for Xbp1, HERP, CHOP, GRP78, and GAPDH has been described (Bergeron et al. 2013); p53-forward: 5'-CTCTCCTCAACAAGTTGTTTTG-3', p53-reverse: 5'-CTACAGTCAGAGCAGCGTTCATGG-3', p21-forward: 5'-GCAGACCAGCATGACAGATTT-3', p21-reverse: 5'-GGATTAGGGCTTCCTCTTRGA-3', PIG3-forward: 5'-AMTGTCAGAGACAAGGCCRRTA-3', PIG3-reverse: 5'-TCCCCRATCTTCCAGTGYCC-3', NOTCH1-forward: 5'-GAACTGCCCATGACCACTACCCAGTTC-3', NOTCH1-reverse: 5'-GGGTGTTGTCCACAGGTGA-3', All qRT-PCR reactions satisfied MIQE guidelines (Bustin et al. 2009): Disassociation profiles in reactions that yielded products contained single homogeneous peaks. In all reactions GAPDH was used as a normalizer. In previous experiments (Bodnarchuk et al. 2012) qRT-PCR arrays comparing Zhangfei

expressing and non-expressing cells five housekeeping genes were analyzed. The levels of GAPDH were not affected by Zhangfei expression.

4.3.8 Co-immunoprecipitation

U2OS and MG63 cells in 6-well dishes were infected with Adeno-ZF or mock infected. Twenty-four hr after infection, cells were washed with cold PBS and lysed in 250µl/well cold lysis buffer (50mM Tris, pH7.5, 150mM NaCl, 1mM EDTA and 0.1% TritonX-100) containing protease inhibitor cocktail (Sigma, P8340). After centrifugation at 13,000 xg at 4 ^{o}C, 20µl of cell lysate supernatant were frozen as pre-immunoprecipitated sample, and mouse anti-p53 antibody (2µg, Santa Cruz Biotechnology, DO-1 sc-126) was added to the remaining supernatant (230µl) and the sample incubated for 12hr with constant gentle agitation. Protein A/G agarose beads (100µl, Pierce, Fisher Scientific, 20421) was added and the samples were incubated for an additional 4hr at 4 ^{o}C. Agarose beads were collected by centrifugation at 13,000 xg at 4 ^{o}C and washed 4 times in lysis buffer before boiling in SDS-PAGE sample buffer. Proteins in samples of the unfractionated cell lysate or immunoprecipitates were separated by SDS-PAGE, transferred to membranes and probed with rabbit anti-Zhangfei antisera, mouse anti-p53, or rabbit anti-mdm2 (Santa Cruz Biotechnology, C-18 sc-821). Antibodies were visualized after incubation with Alexa488-labelled anti-rabbit or anti-mouse antibody.

4.3.9 Statistical analysis

Statistical analysis was preformed by T-test or ANOVA test using IBM SPSS statistics version 21.0.0 software. ANOVA tests with LSDpost hoc comparison was used to analyze the differences between multi-group means and their associated procedures by adding individuals as a treatment variable, and a paired t-test was used to evaluate the effects of one treatment compared with no treatment/control. A *P* value of less than 0.05 was considered to be statistically significant for ANOVA tests and t-tests.

4.4 Results

4.4.1 Leucine-Zipper is required for the suppressive effects of Zhangfei on both cell growth and UPR.

We have previously shown that Zhangfei has a profound effect on the growth and the induction of the UPR in the canine osteosarcoma cell line, D-17 (Chapter 2) (Bergeron et al. 2013). We have also shown that Zhangfei requires its leucine zipper to suppress the UPR in some cells (Chapter 3) (Zhang et al. 2013). To better characterize the molecular mechanism by which Zhangfei suppresses the growth of these cells, we attempted to generate clones of D-17 cells in which Zhangfei could be induced. Six clones that produced stable and detectable Zhangfei only in the presence of the inducer, doxycycline (Fig 4.1A shows evidence from representative clones), were analyzed further. While the original D-17 cells responded to the ectopic expression of Zhangfei by complete cessation of growth [Fig 4.1B, compares cells infected with adenovirus vectors expressing either Zhangfei (Adeno-ZF) or the control protein β-galactosidase (Adeno-LacZ) and mock-infected cells (MI)] the induction of Zhangfei had little or no effect on most of the clones (Fig 4.1B, clones 4, 9, 11, 12 and 16). The induction of Zhangfei had a partial effect on growth on clone 15. We next amplified the complete coding sequences for Zhangfei by PCR from RNA purified from the induced cell clones as well as from pTRE-tightZF (the plasmid used to develop the doxycycline-inducible clones) and determined the nucleotide sequence of the products. While the derived amino acid sequence of the leucine zipper (LZip) region from pTRE-TightZF was identical to the published sequence, one or more leucines from the LZip regions of all clones had been replaced with other amino acids (Fig 4.1C, bold). No other changes were observed in the entire Zhangfei coding sequences suggesting that the leucine mutations were not random changes. To determine if the Zhangfei mutants retained the ability to suppress the UPR, we induced the UPR in the mutant cell clones with thapsigargin, with and without doxycycline, and compared stable levels of transcripts for four UPR related genes—Xbp1s, HERP, CHOP and GRP78. As controls, we compared the original D-17 cells, mock-infected or infected with Adeno-ZF. While Zhangfei suppressed the UPR transcripts in infected cells, doxycycline-induction of the protein in the cell clones had no

effect on them Fig 4.1D). These results suggest that Zhangfei requires a functional leucine zipper for suppressing cell growth as well as inhibiting the UPR.

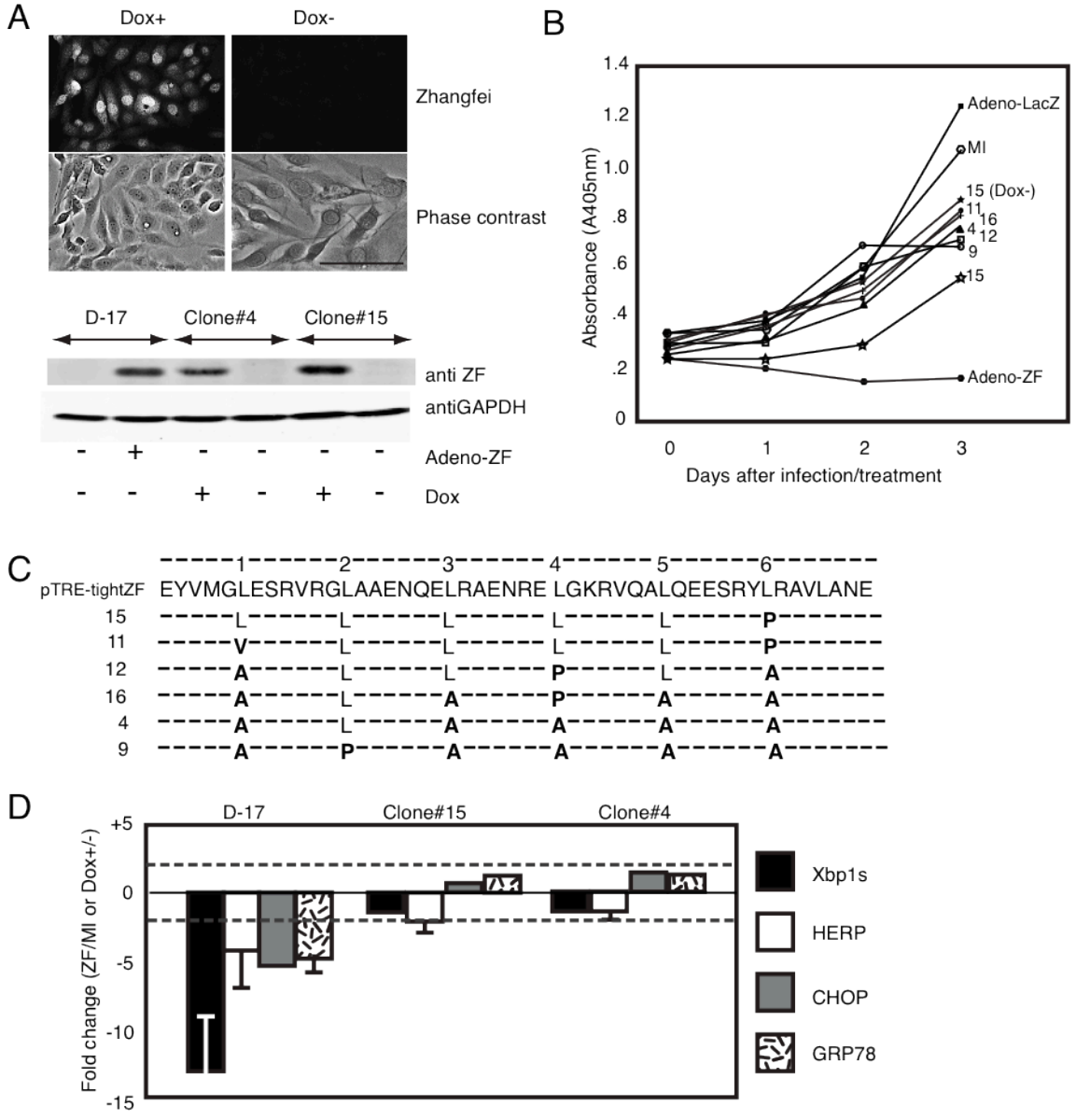

Figure 4.1 Spontaneous mutation of leucine residues in the bLZip domain of Zhangfei in D-17 cells stably expressing the protein in the presence of tetracycline. (A) Doxycycline induces the expression of Zhangfei protein in D-17 cell clones. Twenty four hours after induction with 1µg/ml doxycycline, Zhangfei protein in D-17 clones was detected by immunofluorescence and immunoblotting. Bar=100µm. (B) Growth of D-17 cells after induction of Zhangfei. Six D-17 clones were treated with 1µg/ml doxycycline

to induce Zhangfei expression. Cell growth characteristics were monitored at different time points using WST-1 assay. Growth characteristics of the original D-17 cells, mock-infected (MI) or infected with adenovirus vectors expressing either ZF (Adeno-ZF) or beta-galactosidase (Adeno-LacZ) are shown for comparison. (C) Mutations in the leucine zipper of Zhangfei-expressing D-17 clones. Amino acid sequence of the LZip region for Zhangfei recovered by PCR from the vector used to develop the clones and the doxycycline-inducible Zhangfei expressing clones. Dashes (-) indicate no changes. Leucine residues (L) comprising the zipper are shown and mutations are in bold. (D) Induction of mutant Zhangfei has no effect on the UPR. Clones expressing ZF with mutations in the leucine zipper did not inhibit the UPR. Original D-17 cells and clones 4 and 15 were treated with 1μg/ml doxycycline for 24h, followed by 4h of treatment of thapsigargin. Then cells were harvested and UPR transcripts were estimated by qRT-PCR. The original D-17 cells, mock-infected or infected with Adeno-ZF were also analyzed for comparison. The values represent fold changes in levels of transcripts between Adeno-ZF infected cells with mock-infected cells (in original D-17 cells) or between doxycycline treated with untreated cells (in Tet-on clones). Error bars indicated standard deviations from means of three individual experiments. Dashed lines indicate fold changes of less or more than two fold and changes exceeding these limits were arbitrarily regarded as significant.

4.4.2 Zhangfei regulates p53 at a post-translational level and promotes p53 nuclear retention.

Zhangfei stabilizes p53 in some cancer cells (Lopez-Mateo et al. 2012), although the mechanisms by which Zhangfei mediates its effects on p53 have not been characterized. To investigate if Zhangfei regulates the expression of endogenous p53 and its target genes, we infected D-17 cells with Adeno-ZF or Adeno-LacZ. We observed that Zhangfei increased transcripts for p21, a well-characterized p53-target gene, but had no significant effects on p53 transcripts as well as its other target genes, PIG3 and NOTCH1 (Fig 4.2A). However, Zhangfei did increase the protein levels of both endogenous p53 and p21 comparable to that induced by the drug doxorubicin, a known inducer of p53 (Wang et al. 2004) (Fig 4.2B, compare lanes 3 and 4 with 1 and 2), suggesting that Zhangfei increased p53 protein by post-translational mechanisms. We also assessed the ability of Zhangfei to indirectly induce transcription from a promoter containing two copies of p53 responsive elements (pCAT3B-p53RE). Zhangfei induced the expression of a reporter gene, CAT, linked to the promoter by about twenty fold (Fig 4.2C).

The protein p53 possesses nuclear localization and nuclear export signals enabling it to shuttle between the nucleus and the cytoplasm (Shaulsky et al. 1990; Zhang and Xiong 2001). To investigate the impact of Zhangfei on p53 nucleo-cytoplasmic shuttling, we monitored the p53 localization in ZF-expressing D-17 cells. We observed that, compared with the negative control (pcDNA3), the nuclear staining of endogenous p53 increased by 12h after transfection of the cells with a plasmid expressing Zhangfei (pcZF) with a concomitant decrease in cytoplasmic staining (Fig 4.2D). By 36h following transfection, endogenous p53 was predominantly in the nucleus and cells displayed features of apoptosis (diffuse DNA staining by Hoechst and membrane blebbing).

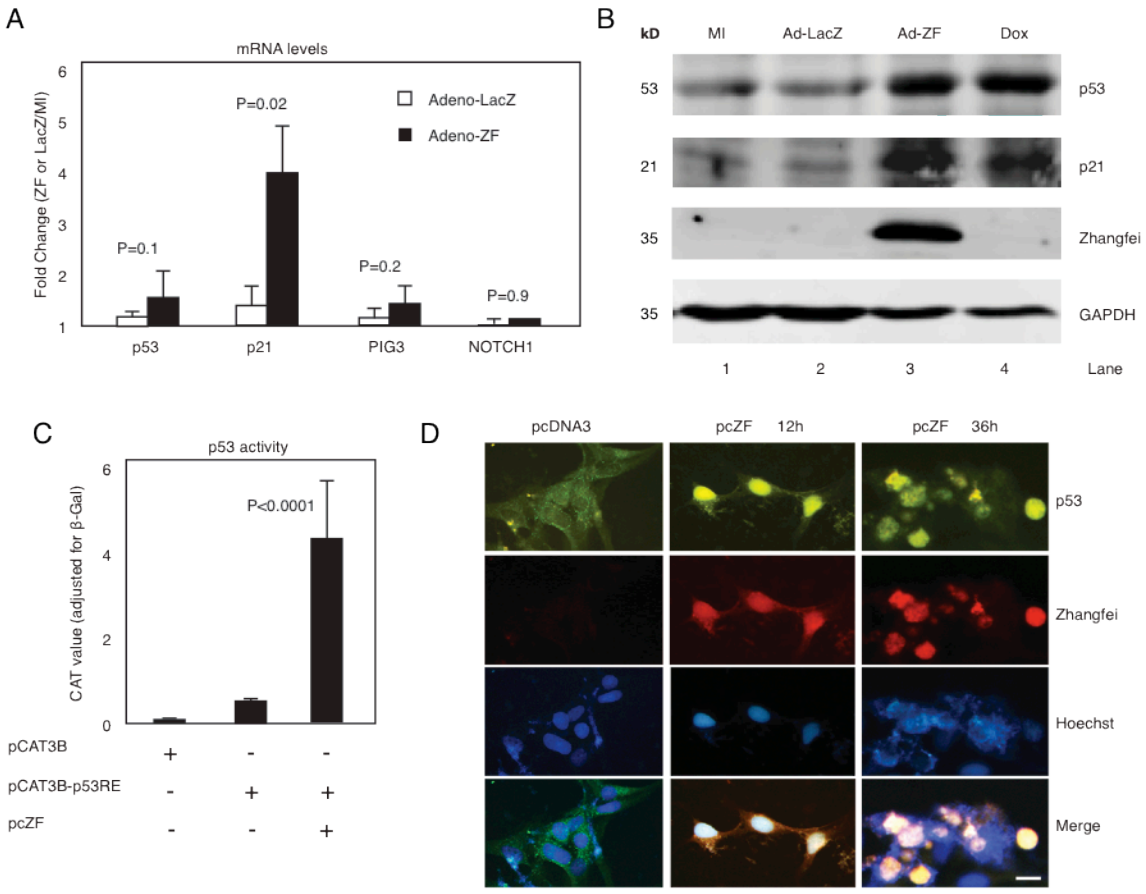

Figure 4.2 Zhangfei regulates p53 in a post-translational level and induces p53 nuclear localization. (A) Zhangfei enhances the expression of p21, a p53-dependant gene. D-17 cells were mock-infected or infected with either Adeno-ZF or Adeno-LacZ, 24 hours after infection, transcripts for p53, p21, PIG3, and NOTCH1 were determined by qRT-PCR. The values represented fold changes of transcripts between Adeno-LacZ (white bar) or Adeno-ZF (black bar) infected cells with mock-infected cells. Error bars indicated standard deviations from means. (B) Zhangfei stabilizes p53 and p21 proteins. p53, p21, Zhangfei and GAPDH were detected by immunoblotting in D-17 cells either mock-infected (MI) or infected with either Adeno-LacZ or Adeno-ZF. As a positive control cells were treated with 0.5μM doxorubicin for 6h. (C) Zhangfei activates p53-dependent transactivation. D-17 cells were transfected with a reporter plasmid containing the coding sequence for CAT linked to a promoter with two copies of p53 responsive element (pCAT3B-p53RE, 0.5μg) in the presence or absence of a plasmid expressing Zhangfei (pcZF, 1μg). The promoter-less parental reporter plasmid, pCAT3B was

included as a control to show basal CAT activity. All samples also contained, as a control, a plasmid expressing b-galactosidase. 24h after transfection, the CAT activity was determined. Values represented the relative CAT activity (normalized to the internal control, b-galactosidase) of different treatments. Standard deviations from means of three individual experiments are shown and the significant *P* values from ANOVA tests were noted above the bars. (D) Zhangfei alters the subcellular localization of p53. D-17 cells were transfected with 1μg of pcZF or a control (pcDNA3), and 12h and 36h after transfection, endogenous p53 as well as Zhangfei were visualized by immunofluorescence with anti-p53 and anti-ZF antibody. The nucleus was detected by Hoechst staining (bar=10μm). The means and standard deviations of representative experiments (n=3) were shown. $P<0.05$ were considered to be significant.

4.4.3 Basic-region leucine zipper domain (bLZip) of Zhangfei is required for the regulation of p53.

Given that the bLZip domain plays an important role in the inhibitory ability of Zhangfei on cell growth and the UPR as described above, we next sought to examine whether this domain was also required for the regulation of p53. We found that transfection of plasmids expressing Zhangfei with a deleted basic region (pcZF Basic del) or a mutated leucine zipper (pcZF Zip(L>A)) (Fig 4.3A) did not increase the protein levels of either p53 or p21 (Fig 4.3B, compare lane 2 with lanes 3 and 4). The increase of p53 transcriptional activity induced by wild-type Zhangfei was also significantly reduced in cells expressing the mutated proteins (Fig 4.3C). In addition, the mutant Zhangfei proteins were unable to increase nuclear localization of p53 (Fig 4.3D). These results indicate that bLZip domain is an important functional region of Zhangfei, required for its regulatory effects on cell growth, the UPR, as well for its interaction with p53.

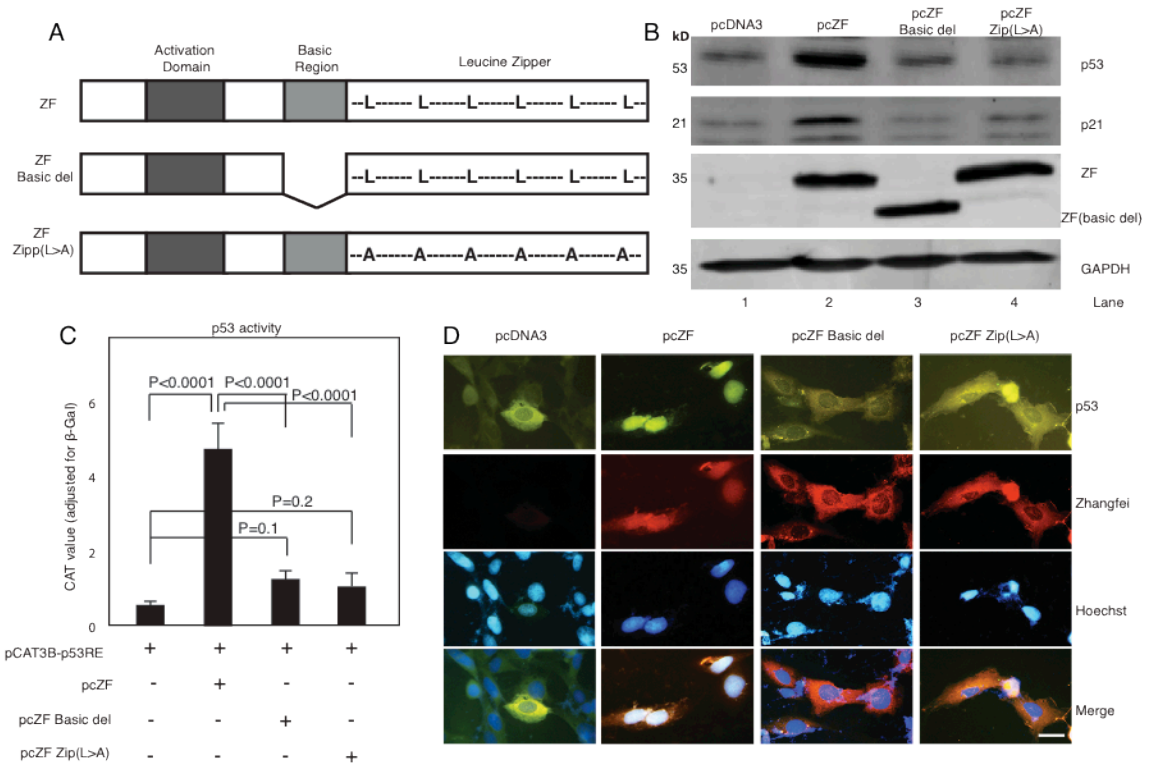

Figure 4.3 The basic-region leucine zipper domain (bLZip) of Zhangfei is required for its effect on p53. (A) Schematic representation of the structures of Zhangfei (ZF) and Zhangfei mutants: ZF Basic del, basic region was deleted; ZF Zip(L>A), all leucines in the leucine-zipper domain were replaced with alanines. (B) The bLZip domain of Zhangfei is required for stabilization of p53 and p21 proteins. D-17 cells were transfected with 1μg of plasmid expressing Zhangfei (pcZF) or mutants (pcZF Zip(L>A) or pcZF Basic del). 24h after transfection endogenous p53 and p21 proteins were detected by immunoblotting. (C) The bLZip domain of Zhangfei is required for p53-dependent transactivation. D-17 cells were transfected with 0.5μg of p53 response element containing reporter plasmid pCAT3B-p53RE and 1μg of pcZF or mutants (pcZF Zip(L>A) or pcZF Basic del). Twenty four hours after transfection, the CAT activity was determined. The means and standard deviations of experiments (n=3) were shown. $P<0.05$ were considered to be significant. (D) The bLZip domain of Zhangfei is required for p53 nuclear retention. D-17 cells were treated as described in (A), and endogenous p53 as well as Zhangfei were visualized by immunofluorescence. The nucleus was detected by Hoechst staining (bar=10μm).

4.4.4 p53 is the key molecule responsible for mediating suppressive regulation of Zhangfei on D-17 cell growth and the UPR.

The tumour suppressor p53 limits cellular proliferation by inducing cell cycle arrest and apoptosis in response to cellular stresses such as DNA damage, hypoxia, nutrient deprivation and oncogene activation (reviewed by (Sharpless and DePinho 2002; Vousden and Lu 2002)), and these stresses also activate the UPR. The results shown above demonstrated that Zhangfei down-regulated cell growth and UPR, but up-regulated p53 through its bLZip domain. To explore whether Zhangfei expression influence cell proliferation and the UPR through p53, we used p53-specific siRNA to knock down p53 transcripts and protein. While Zhangfei enhanced transcription from a promoter with p53 response elements, siRNA against p53 suppressed its effects (Fig 4.4A). Suppression of p53 also partially restored the growth of D-17 cells inhibited by Zhangfei (Fig 4.4B) and partially restored the suppression of UPR transcripts (Fig 4.4C). These results indicate that the inhibitory influences of Zhangfei on D-17 cells that we have described previously, at least in part, are mediated by p53.

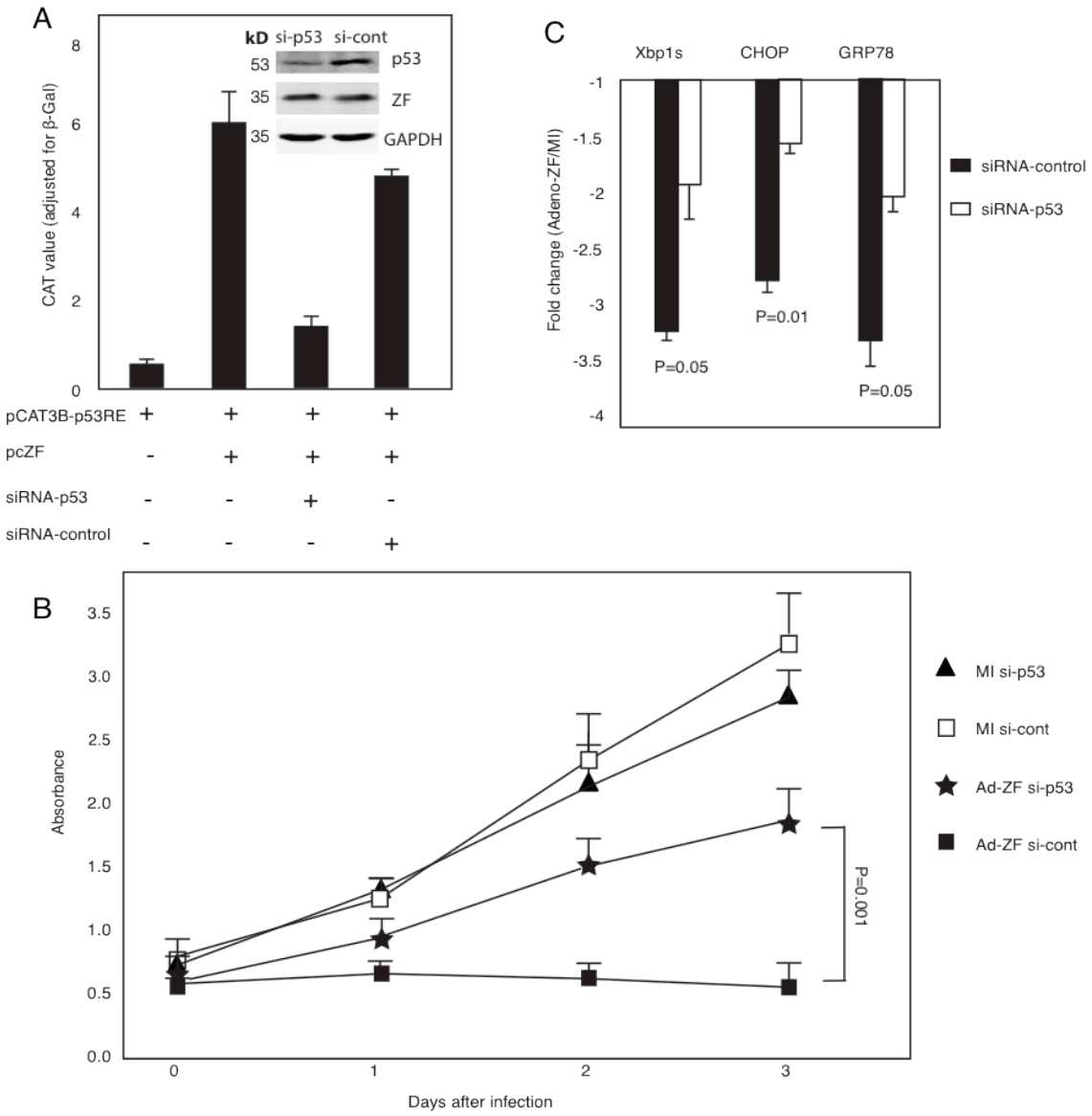

Figure 4.4 Zhangfei regulates p53-mediated cell growth and UPR. (A) Zhangfei-induced transcription from a promoter with p53 response elements is mediated by p53. D-17 cells were transfected with 0.5μg of p53 response element containing promoter (pCAT3B-p53), 1μg of plasmid expressing Zhangfei (pcZF) and 100pM of si-RNA against p53 (si-p53) or non-targeting siRNA control (si-cont), 48h after transfection, cells were analyzed for CAT activity or p53, Zhangfei and GAPDH proteins (inset). (B) Zhangfei-mediated suppression of cell growth is mediated through p53. D-17 cells were transfected with 100pM of si-RNA against p53 (siRNA-p53) or non-targeting siRNA control (siRNA-cont). Eight hours after transfection, cells were either mock-infected or

infected with Adeno-ZF, and growth characteristics were measured using WST-1 assay at 0h, 24h, 48h and 72h after infection. (C) Zhangfei-mediated suppression of UPR genes is mediated by p53. D-17 cells were transfected 100pM of siRNA-p53 or non-targeting siRNA-control. 48h after transfection, cells were treated with thapsigargin for 4h and harvested. Differences in levels of transcripts (Xbp1s, CHOP, and GRP78) were determined by qRT-PCR. The results were expressed as fold changes relative to mock-infected cells. The means and standard deviations of experiments (n=3) are shown. $P<0.05$ were considered to be significant.

4.4.5 Zhangfei suppresses the growth and UPR in p53-expressing, but not in p53-null human osteosarcoma cells.

To determine if our results linking p53 as the mediator of the effects of Zhangfei on canine osteosarcoma cells applied to human osteosarcoma cells as well, we examined two human cells lines—U2OS cells possess and express a functional p53 protein whereas MG63 cells do not (Park et al. 2002). We confirmed this by detecting and comparing p53 transcriptional activities of both cell lines (Fig 4.5A). Both osteosarcoma cell lines have been used extensively to assess the role of p53 in several phenomena.

As with D-17 cells, Zhangfei suppressed the growth of U2OS cells but had little effect on MG63 cells (Fig 4.5C). Immunofluorescent detection of Zhangfei in these cells showed that both cells expressed Zhangfei when infected with an adenovirus vector expressing the protein (Fig 4.5B). Also, as in D-17 cells, ectopic expression of Zhangfei suppressed UPR-related transcripts while increasing levels of p53-related transcripts in U2OS cells but not in MG63 cells (Fig 4.5D, 4.5E). Figure 5F further supports these observations by showing that, as in D-17 cells, in U2OS cells p53 and p21 proteins increased but, as expected, they did not in MG63 cells.

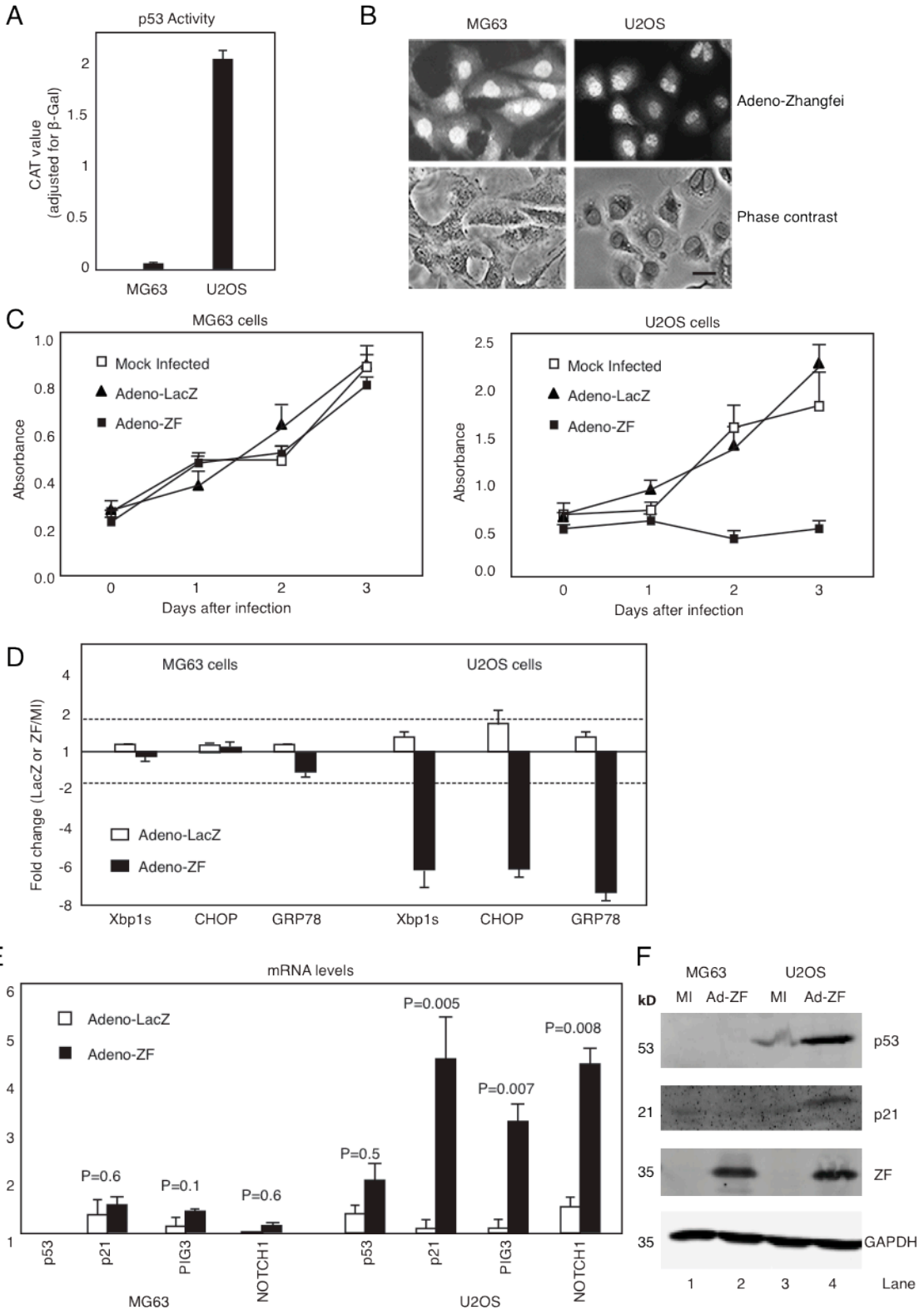

Figure 4.5 Zhangfei suppresses cell growth and UPR in wild-type p53-expressing U2OS cells, but not in p53-null MG63 cells. (A) p53-dependent transactivation in

U2OS and MG63 cells. Cells were transfected with 0.5μg of p53 reporter (pCAT3B-p53RE) for 24h, then p53 transcriptional activity was determined by CAT ELISA assay. (B) U2OS and MG63 cells express Zhangfei when infected with adenovirus vector expressing the protein. Cells were infected with Adeno-ZF, and 24h later Zhangfei protein was visualized by immunofluorescence (bar=10μm). (C) Ectopic expression of ZF suppresses cell growth in p53-wild type U2OS cells, but has no effect in p53-null MG63 cells. U2OS and MG63 human osteosarcoma cells were mock-infected or infected with Adeno-ZF or Adeno-LacZ and their growth rates were assessed at different time points after infection as absorbance at 405 nm after treatment with WST-1. (D) Zhangfei negatively regulates the UPR in U2OS cells but not in MG63 cells. U2OS and MG63 human cells were either mock-infected or infected with Adeno-ZF or Adeno-LacZ. 24h after infection, cells were treated with thapsigargin for 4h, and then harvested. The differences in levels of transcripts for UPR genes (Xbp1s, CHOP, and GRP78) were determined by qRT-PCR. (E) Zhangfei enhances p53-dependent transcripts in U2OS cells but not in MG63 cells. Transcripts for p53, p21, PIG3, and NOTCH1 in U2OS and MG63 cells either mock-infected or infected with Adeno-LacZ or Adeno-ZF were measured by pRT-PCR. (F) Zhangfei increases p53 and p21 proteins in U2OS cells but not in MG63 cells. p53 and p21 proteins were detected by immunoblotting in U2OS and MG63 cells either mock-infected or infected with Adeno-ZF. The values in (D) and (E) represented fold changes of transcripts between Adeno-LacZ (white bar) or Adeno-ZF (black bar) infected cells with mock infected cells. The means and standard deviations of experiments (n=3) were shown. $P<0.05$ were considered to be significant. Error bars indicate standard deviations from means. Horizontal dotted line indicated a two-fold change. A change of more than 2-fold was arbitrarily regarded as significant.

To confirm that the suppressive effects of Zhangfei on growth and UPR of U2OS cells were indeed mediated through p53, we suppressed p53 in these cells with siRNA. Figure 4.6A shows that treatment with siRNA directed against p53 suppressed activation of transcription from a promoter with p53 response elements and reduced levels of p53 protein. The specific suppression of p53 had a significant effect on the ability of Zhangfei to inhibit the growth of U2OS cells (Fig 4.6B) and UPR-related transcripts (Fig 4.6C). To further evaluate the role of p53 in cell growth arrest and UPR suppression induced by Zhangfei, studies were carried out to determine the effects of introducing p53 using a plasmid expressing wild-type p53 (from U2OS) into p53-null MG63 human cells in absence or presence of Zhangfei. The overexpression of exogenous p53 (confirmed by immunoblotting and CAT reporter assay in Fig 4.6D) had a strong inhibitory effect on cell growth and led to cell death within 24-36 hours (Fig 4.6E, open squares), which is consistent with a previous report that the infection of adenovirus vector expressing wild-type p53 in p53-null Saos-2 osteosarcoma cells induced these cells to commit apoptosis (Marcellus et al. 1996), and Zhangfei further increased this suppression (Fig 4.6E, solid squares). In contrast, Zhangfei alone had no effect on cell growth in these cells (Fig 4.6E, stars). For the influence on UPR, p53 expression in MG63 cells led to decreases (about 1.5-2.5 folds) in the levels of UPR transcripts induced by thapsigargin (Fig 4.6F, black bars). And ectopic expression of Zhangfei suppressed UPR-related transcripts more potently (about 3.5-5 fold decrease) in p53-expressing MG63 cells than that in p53-null MG63 cells (Fig 4.6F), suggesting p53 acts as an intermediary in the suppressive events induced by Zhangfei.

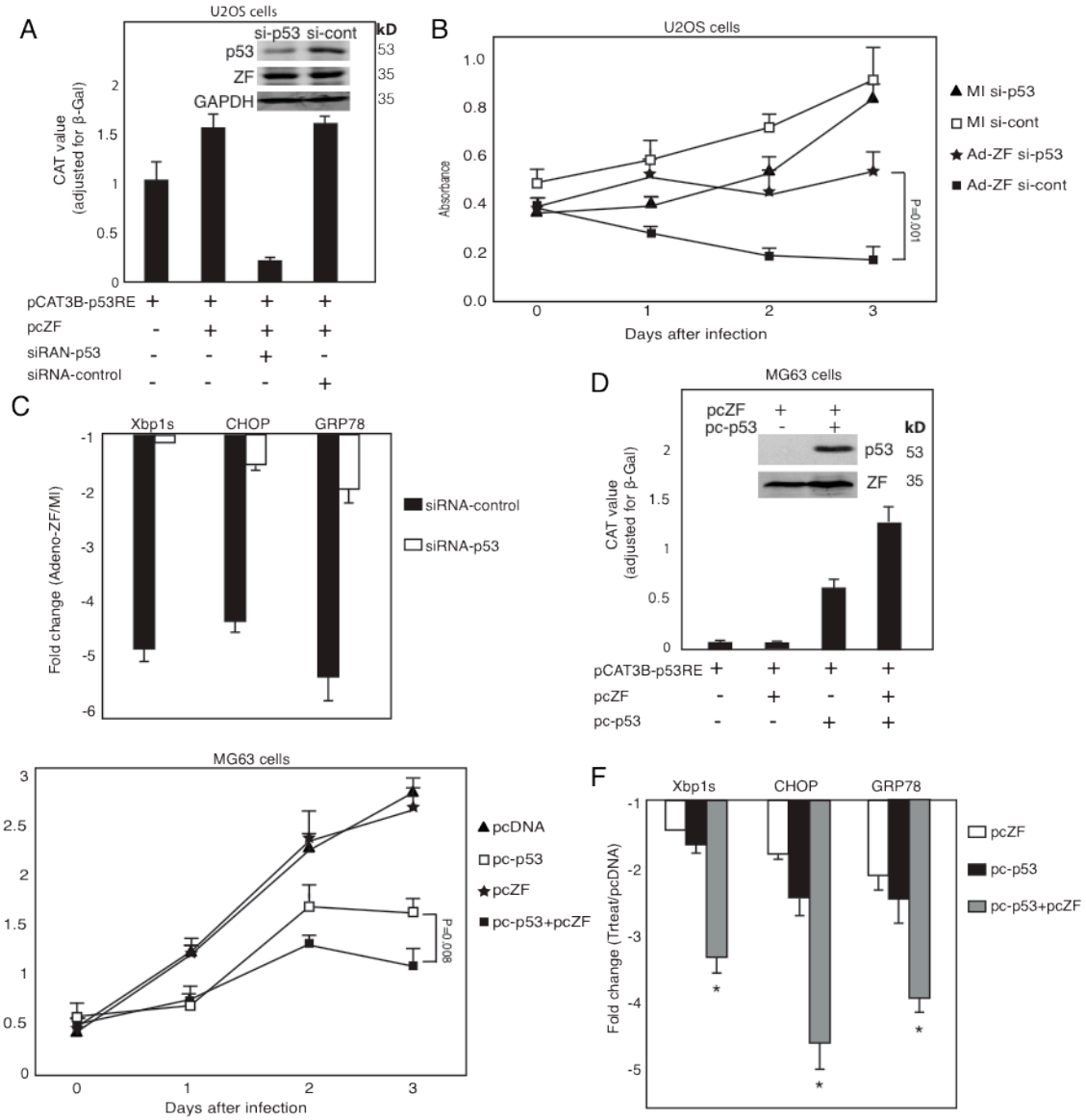

Figure 4.6 p53 mediates the suppressive effects of Zhangfei on cell growth and UPR in human osteosarcoma cells. (A) Zhangfei-mediated transcription from a p53 response element-containing promoter is mediated by p53 in U2OS cells. U2OS cells were transfected with 0.5μg of p53 reporter plasmid (pCAT3B-p53RE) and 1μg of plasmids expressing Zhangfei (pcZF) in the presence of 100pM of siRNA-p53 or non-targeting siRNA-control. Forty eight hours after transfection CAT reporter activity and p53 protein were detected by ELISA and immunoblotting (inset). (B) Suppression of p53 by siRNA in U2OS cells restored Zhangfei-induced suppression of cell growth. U2OS were transfected with 100pM of siRNA-p53 or non-targeting siRNA-control. Eight hours after

transfection, cells were either mock-infected or infected with Adeno-ZF and measured growth rates as absorbance at 405 nm after treatment with WST-1. (C) Suppression of p53 prevents Zhangfei-induced inhibition of UPR genes. U2OS cells were transfected with 100pM of siRNA-p53 or non-targeting siRNA-control. Forty eight hours after transfection, cells were treated with thapsigargin for 4h and harvested. Differences in levels of transcripts for UPR genes (Xbp1s, CHOP, and GRP78) were determined by qRT-PCR. The values represented fold changes of transcripts between Adeno-LacZ (white bar) or Adeno-ZF (black bar) infected cells with mock infected cells. (D) Transactivation activity and expression of p53 protein in p53-null MG63 cells. p53-null MG63 cells were transfected with 0.5μg of p53 reporter plasmid and 1μg of plasmids expressing Zhangfei (pcZF) in the presence of 1μg of plasmids expressing wild-type p53 (pc-p53, from U2OS cells) for 24h, and then CAT reporter activity and p53 protein were detected by ELISA and immunoblotting. (E) Overexpression of p53 in p53-null MG63 cells suppressed cell growth and activated growth arrest induced by Zhangfei. MG63 cells were transfected with 3μg of pcZF in the absence or presence of 3μg of pc-p53 and measured growth rates as absorbance at 405 nm after treatment with WST-1. (F) Overexpression of p53 in p53-null MG63 cells activated the UPR suppression induced by Zhangfei. MG63 cells were transfected with 3μg of pcZF in the absence or presence of 3μg of pc-p53. Twenty four hours after transfection, cells were treated with thapsigargin for 4h and harvested. Differences in levels of transcripts for UPR genes (Xbp1s, CHOP, and GRP78) were determined by qRT-PCR. The values represented fold changes of transcripts between pcZF (white bar), pc-p53 (black bar), or pcZF plus pc-p53 (grey bar) transfected cells with pcDNA3 transfected cells. The total amount of DNA in each transfection above was made up to 5 μg with pcDNA3. The means and standard deviations of experiments (n=3) were shown. *P<0.05 were considered to be significant.

4.4.6 Zhangfei interacts with p53.

Next, we confirmed that p53 and Zhangfei interacted in U2OS cells by co-immunoprecipitating the proteins in cells expressing Zhangfei. Immunoprecipitation of p53 from U2OS cells also precipitated Zhangfei (Fig 4.7A). As expected, in MG63 cells, which lack p53, no p53 was detected and, consequently, no Zhangfei was precipitated.

4.4.7 Zhangfei displaces mdm2 from p53, protecting it from proteolysis.

In unstressed cells p53 is associated with the E3 ubiquitin ligase, mouse double minute homologue 2 (mdm2) that facilitates its nuclear export (Roth et al. 1998; Carter et al. 2007)and proteasomal degradation (Honda et al. 1997). To determine if Zhangfei inhibited mdm2-p53 interactions, we immunoprecipitated p53 from mock-infected and Adeno-ZF infected U2OS cells and probed the precipitates for mdm2 and Zhangfei (Fig 4.7B). p53 was precipitated from both mock-infected and Zhangfei-expressing cells but it was only associated with mdm2 in the absence of Zhangfei. In addition, while the proteasome inhibitor MG132 (5μM) prevented the degradation of p53 in mock-infected cells it had little effect on p53 when Zhangfei was present (Fig 4.7C). These results indicate that Zhangfei displaces mdm2 from p53 thereby preventing mdm2-mediated nuclear export and subsequent proteasomal degradation of p53 (Fig 4.7D).

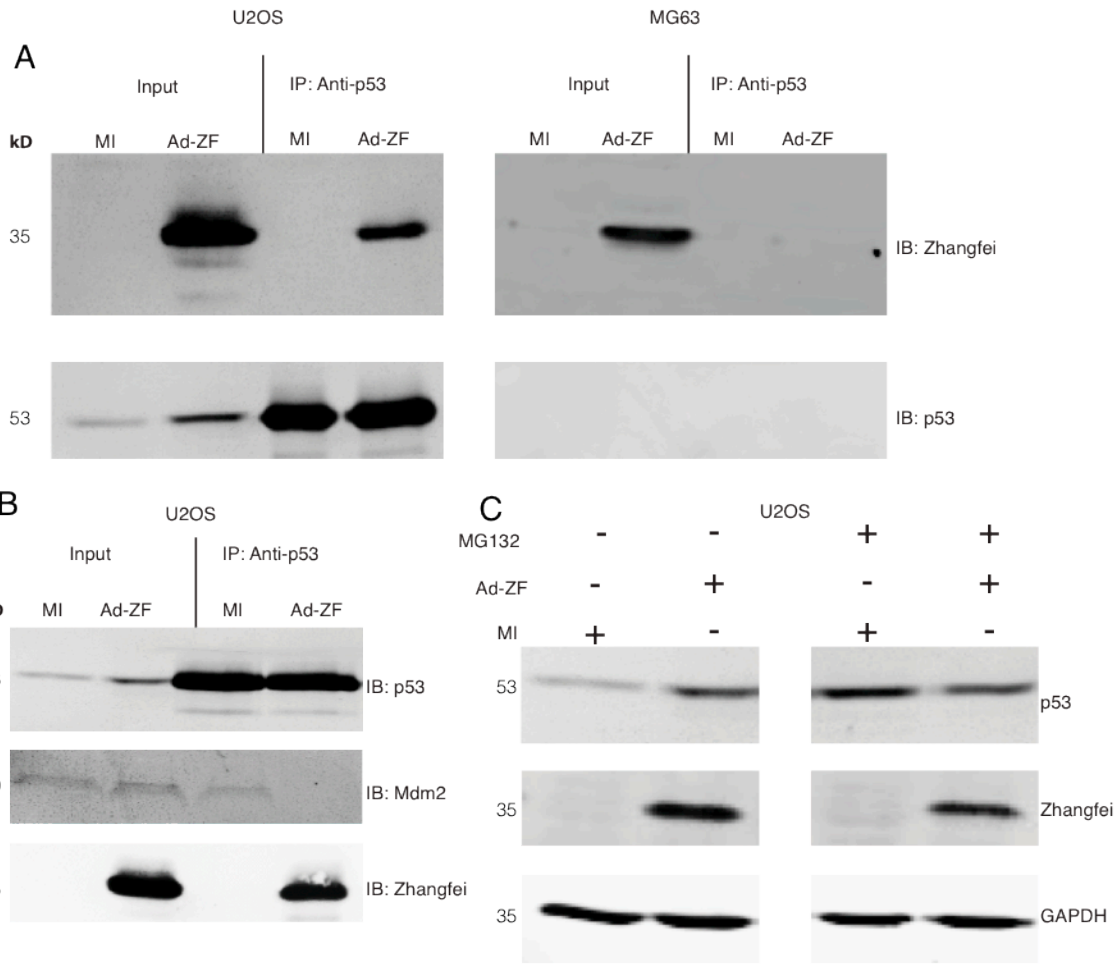
A
U2OS
MG63
Input
IP: Anti-p53
kD
MI
Ad-ZF
35
53
IB: Zhangfei
IB: p53
B
U2OS
60
IB: Mdm2
C
MG132
p53
GAPDH
D
Normal conditions
Zhangfei involved
Ub
p53 protein
26S proteasome
Cytoplasm
Nuclear
Mdm2
Target genes
Activation
ZF
Cell growth arrest
UPR suppression

Figure 4.7 In vitro interaction of Zhangfei and p53. (A) Zhangfei associates with p53 in U2OS cells. Cell lysate from U2OS and MG63 human cells previously infected with adenovirus vector expressing Zhangfei (Ad-ZF) or mock infected (MI) were incubated with 2μg of mouse anti-p53 antibody for 12 hours at 4 ^{o}C, followed by an additional 4 hours incubation with 100μl of Protein A/G agarose beads (IP). The associated proteins were detected by immune-blotting (IB) using rabbit anti-Zhangfei antibody and mouse anti-p53 antibody. Input represented 1/10 of lysate used for immunoprecipitation. (B) Zhangfei displaces mdm2 from p53 in U2OS cells. Cell lysates as in (A) were immunoprecipitated with anti-p53 antibody followed by immunoblotting with either anti-mdm2 or anti-Zhangfei antibody. (C) Inhibition of proteasomal degradation reverses degradation of p53 in the absence of Zhangfei. Cells infected as in (A) were either treated with the proteasomal inhibitor MG132 (5μM) or left untreated. Proteins in the Cell lysates were separated by SDS-PAGE and p53, Zhangfei and GAPDH (loading control) were detected by immunoblotting. (D) Schematic diagram of a model for the proposed mechanism by which Zhangfei inhibits cell growth and UPR. Under normal conditions, mdm2 translocates the p53 protein out of the nucleus for degradation via the ubiquitin-dependent pathway. In Zhangfei-expressing cells, Zhangfei interacts with p53 and prevents it from binding to mdm2. This results in p53 stabilization and nuclear accumulation, which, in turn, activates its target genes and suppresses tumour cell growth and the UPR. These results illustrate how Zhangfei controls the activity of p53 toward the cell growth and the UPR, and offer an explanation to why p53 is upregulated by Zhangfei in tumour cells.

4.5 Discussion

The protein p53 is a key tumour suppressor protein. Various cellular stresses, such as UV radiation, DNA damage, hypoxia, and oncogene activation, activate p53, which functions as a transcription factor, regulating the expression of a large and disparate group of target genes to initiate apoptosis, cell cycle arrest, DNA-repair, cellular senescence as well as differentiation. To overcome this suppression, tumour development is often accompanied by mutation or loss of p53. The transcription factor Zhangfei activates the mitogen-activated protein kinase (MAPK) pathway that directs medulloblastoma cells to commit apoptosis (Bodnarchuk et al. 2012). In addition to this pathway, here we demonstrate another tumour repressor role for Zhangfei through its ability to directly interact with tumour suppressor p53, and to promote p53 protein stabilization and its nuclear retention in canine and human osteosarcoma cells. Of note the knockdown of endogenous p53, partly but significantly, counteracted Zhangfei-induced arrest of cell growth in p53-wild type osteosarcoma cells (D-17 and U2OS), while exogenous expression of p53 enhanced this process in p53-null osteosarcoma (MG63).

In normal cells, p53 is a short-lived protein and functions to control excessive cell proliferation. Under unstressed conditions low intranuclear concentrations of p53 protein are maintained by its binding to E3 ubiquitin-ligases such as mdm2, COP1 and pirh2, which keep p53 in check by ubiqitination, nuclear export, and proteasomal degradation (reviewed by (Vousden and Lane 2007)). In our studies, we found that the suppressive effect of Zhangfei on the growth of cells was not universal. In some normal cells, such as MRC5 fibroblasts (Valderrama et al. 2009), or p53-null tumour cells such as MG63 (Fig 4.5C), Zhangfei had no effect on cell proliferation. This selective effect of Zhangfei could be due either to the tight regulation of p53 by means of post-translational modifications, cofactor binding, and subcellular localization, or due to a lack of functional p53. As shown here, the growth of U2OS osteosarcoma cells were dramatically inhibited by Zhangfei (Fig 4.5C and 4.6B), likely because the cells express wild-type p53. However, our results are not in agreement with those of Lopez-Mateo and others, who in a recent study (Lopez-Mateo et al. 2012) showed that tetracyclin-induction of Zhangfei/CREBH in inducible clones derived from U2OS cells resulted in no effect on the proliferation of

the cells, nor did the cells display obvious morphological changes. We speculate that there could be two reasons for the differences in our results: 1) Leaky expression of tetracycline-inducible Zhangfei in U2OS cells (Lopez-Mateo et al. 2012) may have resulted in the selection of clones with structural and functional mutations in Zhangfei. This would be consistent with our observations (Fig 4.1) that suggest that the growth suppressive effects of Zhangfei in cells expressing functional p53 exert strong selective pressure and lead to the selection of cells expressing non-functional Zhangfei. 2) The cell line studied by Lopez and co-workers may have spontaneously lost its functional p53. We confirmed that the U2OS cell line we used in this study does indeed have a functional p53 (Fig 4.5A).

The protein p53 has been reported to antagonize the UPR and inhibit ground glass hepatocyte development during ER stress (Dioufa et al. 2012). In the present study, we determined that p53, at least in part, mediates Zhangfei-induced suppression of the UPR (Fig 4.4 C, 4.6 C and F). Induction of UPR is a protective mechanism utilized by cells to adapt to ER stress. In normal cells, the UPR is related to tissue preservation or organ protection against ER stress. In neoplastic cells, however, the adaptive function of the UPR is implicated in immune resistance, cancer progression, and drug resistance (reviewed by (Moenner et al. 2007; Ron and Walter 2007)). Therefore, the inhibition of UPR, mediated by p53, represents a potential strategy by which Zhangfei could be used to inhibit cancer. We show that Zhangfei inhibits the UPR and induces apoptosis by promoting p53 stabilization and nuclear retention. Interestingly, in keeping with our results, ER stress, and the resulting activation of the UPR induces p53 cytoplasmic localization and prevents p53-dependent apoptosis (Qu et al. 2004), suggesting the existence of an ER stress-p53-UPR regulatory loop (see model in Fig 4.8). Although the precise relationship between p53 stabilization and UPR remains unclear, glycogen synthase kinase-3 is shown to be a mediator of ER-stress induced p53 cytoplasmic localization (Qu et al. 2004), and the ubiquitination and importin-a3 binding of p53 are also associated with stress-mediated p53 translocalization (Becker et al. 2007; Marchenko et al. 2012). In the case of Zhangfei, improved understanding of how exactly p53 suppresses the UPR still should be further investigated.

We had previously shown that ectopic expression of Zhangfei in medulloblastoma (Valderrama et al. 2009) and osteosarcoma cells (Chapter 2) (Bergeron et al. 2013) suppressed the cell growth and the UPR. In addition, the effects of this protein were also observed in four other canine osteosarcoma cell lines (not published, see Chapter 6). To study the mechanisms involved we tried to develop cell lines in which the expression of Zhangfei could be induced by doxycycline. We were successful in developing several clones that expressed Zhangfei protein, as detected by immunofluorescence and immunoblots, only in the presence of the inducer (Fig 4.1A). However, none of the clones behaved as did cells in which the ectopic expression of Zhangfei was induced by infection with adenovirus vectors (Fig 4.1B and D). On further examination we found that in the clones one to as many as all six leucines had been replaced with other amino acids (Fig 4.1C). The clone with only one substitution had growth properties most similar to the cells expressing adenovirus-Zhangfei. No other mutations in the entire Zhangfei coding sequences were observed in any of the clones. We interpret this to mean that even small amounts of intact Zhangfei, that might be expressed in the absence of the inducer, were sufficient to block cell division; only cells in which the zipper had been disabled survived.

While our results clearly identify the leucine zipper of Zhangfei as important for its interaction with p53 and subsequent p53-mediated suppression of the UPR, we have also shown (Chapter 3) that Zhangfei interacts with the bLZip UPR transcription factor Xbp1s, targeting it for proteosomal degradation (Zhang et al. 2013), and that the leucine zipper of Zhangfei is also important for this interaction. Hence, modulation of UPR by Zhangfei may require a combination of several coordinated events, including direct interaction with UPR mediators and indirect mediation through p53.

Like Zhangfei several other bLZip proteins such as ATF3 (Yan et al. 2005; Wang et al. 2010), ING2 (Wang et al. 2006b), K-bLZip (Park et al. 2000), and CREB (Okoshi et al. 2011), are known to interact with p53 through their leucine zippers. The nature of these interactions as well as their consequences, however, varies. Interactions between p53 and Zhangfei, ATF3 and CREB lead to enhanced transcriptional activity and stabilization of p53. Our results show that Zhangfei displaced mdm2 from p53 (Fig 4.7B) and prevented

its degradation to a similar extent as the proteasomal inhibitor MG132 (Fig 4.7C). The effects of MG132 and Zhangfei were not additive. This may reflect the toxic effects of the combined inhibitors leading to a decrease in a rapidly turned over protein like p53. In contrast to the effects of Zhangfei on p53, ATF3 neither displaces mdm2 nor does it suppress its activity. ATF3 binds directly to the carboxyl terminus of p53 and prevents ubiquitination of key lysine residues in the region (Yan et al. 2005). CREB binds to the amino terminus of p53 and acts as a bridge directing CREB binding protein to p53 responsive promoters (Giebler et al. 2000) and suppresses transcription of mdm2 (Okoshi et al. 2008). Unlike the bLZip-p53 interactions mentioned above, which enhance p53 activity, the association of the Kaposi sarcoma herpesvirus protein K-bLZip with p53 suppresses its activity and may contribute to tumorigenesis by the virus (Park et al. 2000).

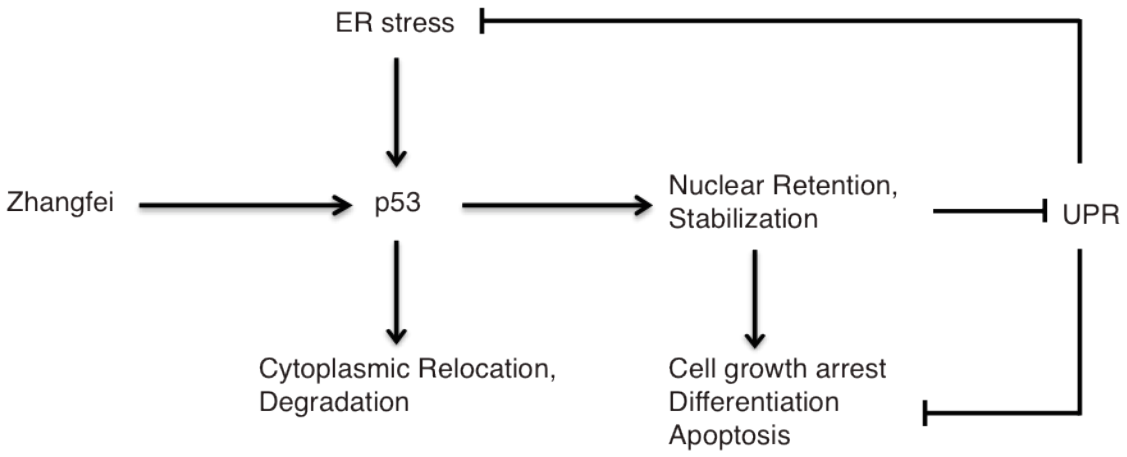

Figure 4.8 Zhangfei and ER stress have opposing effects on p53. In Zhangfei-expressing cells, p53 is targeted for nuclear localization and stabilization, and further to prevent cell growth, induce cell apoptosis, as well as inhibit the UPR in response to ER stress. In contrast, ER stress promotes the cytoplasmic localization and degradation of p53.

4.6 Acknowledgements

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5. Structural domains responsible for p53-Zhangfei interaction

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This Chapter described the characterization of the interaction of Zhangfei and p53 by mapping the interacting domains of p53. It showed that p53 formed a complex with Zhangfei via its N-terminal Transactivation Domain (NTD), which was required for Zhangfei-mediated nuclear retention and transcriptional activity of p53. This Chapter will be submitted in the future with further study for publication as "Characteristics of the interaction of p53 and Zhangfei/CREBZF."

My contributions to this Chapter include: To date, I constructed several plasmids expressing p53 deletion mutants containing its different functional domains, respectively. I also used these constructed plasmids to determine the interacting and transactivating domain of p53 with Zhangfei. The specific amino acid sites of p53 that function as the binding sites of Zhangfei will be detected in future studies using direct-site mutagenesis of p53. I generated all the final figures and wrote the first draft, which was revised by Professor Vikram Misra.

5.1 Abstract

We described (Chapter 4) that basic-region leucine zipper (bLZip) transcriptional factor Zhangfei has the ability to stabilize tumour suppressor p53 and activate its transcription activity by competing with the negative regulator mdm2 which, when bound to p53, targets it for degradation. The bLZip domain of Zhangfei is required for its interaction with p53. In this study, we identified the domains of p53 which are responsible for complex formation with Zhangfei. Immunoprecipitation assay revealed that p53 interacted with Zhangfei through its N-terminal transactivation domain (NTD), which is also the mdm2-binding domain and transcription activation region of p53. Overexpression of Zhangfei enhanced the nuclear retention and increased the transcription activity of both wild-type p53 and the 92 amino acid residues at its N-terminus (NTD region). In contrast, with the mutant form of p53 lacking an NTD region, we could not observe any changes in its cellular localization or transcription activity between the absence and presence of Zhangfei. Our results suggested that Zhangfei positively regulates p53 by occupying the NTD region and thereby preventing its association with negative regulator mdm2.

Key words: p53, Zhangfei, N-terminal transactivation domain, interaction

5.2 Introduction

Tumour suppressor protein p53 plays an important role in cell fate determination in response to oncogenic or other cellular stress signals. It exerts its function as a nuclear sequence-specific transcription factor, and induces or represses the expression of various target genes involved in cell cycle arrest and apoptosis (Joerger and Fersht 2008). p53 comprises several functional regions: the N-terminal transactivation region consists of a transactivation domain (TAD) and a proline-rich region (PRR), followed by the DNA-binding core domain (p53C), a tetramerization domain (TET) and a C terminus (CT) (Fig. 1A). Under physiological conditions, p53 is a short-lived protein that is strictly regulated by several other proteins, and one of the most important of these is E3 ubiquitin-ligase mdm2. The N-terminal sequence-specific transactivation domain (NTD) of p53 is responsible for its transcriptional and pro-apoptotic activities. Thus, mdm2 keeps p53 in check by binding to its NTD, inhibiting its transcriptional and pro-apoptotic activity (Momand et al., 1992), and further targeting p53 for proteasomal degradation via ubiquitin-dependent pathways (Kulikov et al. 2010). In addition, p53 can up-regulate the expression of mdm2 by an auto-regulatory feedback loop to prevent inappropriate apoptosis. Under cellular stress conditions, p53 is rapidly phosphorylated on multiple sites, such as Ser15, Thr18 or Ser20. A potential outcome of such phosphorylation is the stabilization of p53 through disruption of its binding with mdm2 and inhibition of p53 ubiquitination and degradation. Once p53 becomes activated, it functions as a transcription factor and drives the expression of a wide network of apoptotic signals.

Recently, our lab and others have found that the activity of p53 was enhanced by a basic-region leucine zipper (bLZip) transcriptional factor, Zhangfei. Further studies demonstrated that Zhangfei stabilized p53 and promoted its nuclear retention by disrupting its association of mdm2 and preventing p53 from degradation. The bLZip domain of Zhangfei contributed to its profound effects on p53 (Chapter 4) (Lopez-Mateo et al. 2012; Zhang and Misra 2014). The objective of this study is to identify structural domain of p53 responsible for the complex formation with Zhangfei. Our results suggest that p53 forms a complex with Zhangfei via its NTD region, which is required for Zhangfei-mediated nuclear retention and transcriptional activity of p53.

5.3 Materials and Methods

5.3.1 Cells and tissue culture

Human MG63 and U2OS osteosarcoma cells were obtained from Dr. Douglas H. Thamm, (Associate Professor of Oncology, Animal Cancer Center, Colorado State University). Vero cells were obtained from the American Type Tissue Culture Collection. All cell lines were grown in Dulbecco's minimal essential medium containing penicillin and streptomycin and 10% newborn calf serum. All media, serum and antibiotics were purchased from Invitrogen.

5.3.2 Plasmids

The construction of pcZF (Lu and Misra 2000b), a plasmid that expresses Zhangfei in mammalian cells, has been described. The CAT reporter plasmid pCAT3B-p53RE was constructed as described before (Chapter 4) (Zhang and Misra 2014). The construction of the plasmids expressing the full length of wild-type p53 as well as its several deletion mutants was performed using an In-Fusion HD Cloning Kit (Clontech, 639648) as described in the manufacturer's instructions. All of these p53-plasmids contained three copies of the FLAG-coding sequence. The cDNA fragment containing the entire coding sequence of p53 was recovered from U2OS cells and its sequence confirmed. The specific primers for p53 and its deletion mutant amplification are summarized in Table 5.1.

Table 5.1 The sequence of primers for p53 and its deletion mutant amplification

Full length of wild-type p53	Forward	5'- GCGGCCGCGAATTCATGGAGGAGCCGCAGTCAGAT-3'
	Reverse	5'- ATCTATCGATGAATTCTCAGTCTGAGTCAGGCCCTTCT-3'
p53 (1-356aa)	Forward	5'- GCGGCCGCGAATTCAATGGAGGAGCCGCAGTCAGAT-3'
	Reverse	5'- ATCTATCGATGAATTCTCACCCAGCCTGGGCATC -3'
p53 (1-292aa)	Forward	5'- GCGGCCGCGAATTCATGGAGGAGCCGCAGTCAGAT-3'
	Reverse	5'- ATCTATCGATGAATTCTCATTTCTTGCGGAGATTCT-3'
p53 (92-393aa)	Forward	5'- TTGCGGCCGCGAATTCACTGTCATCTTCTGTCCCTT-3'
	Reverse	5'- ATCTATCGATGAATTCTCAGTCTGAGTCAGGCCCTTCT-3'
p53 (92-292aa)	Forward	5'- TTGCGGCCGCGAATTCACTGTCATCTTCTGTCCCTT-3'
	Reverse	5'- ATCTATCGATGAATTCTCATTTCTTGCGGAGATTCT -3'
p53 (1-92aa)	Forward	5'- GCGGCCGCGAATTCATGGAGGAGCCGCAGTCAGAT -3'
	Reverse	5'- ATCTATCGATGAATTCTCAGGGCCAGGAGGGGGC -3'

5.3.3 Transfection and CAT Assays

Vero cells were transfected with plasmids using Lipofectamine 2000 (Invitrogen, 11668-019) as described in the manufacturer's instructions. MG63 cells were transfected using MG-63 Transfection Kit (for Osteosarcoma Cells, CRL-1427) (Cedarlane, 6848) as described in the manufacturer's instructions. Chloramphenicol acetyl transferase (CAT) assays have been described earlier (Zhang and Misra 2014). CAT values were detected using an enzyme-linked immunosorbent assay kit (Roche Applied Science, 11-363-727-001).

5.3.4 Co-immunoprecipitation

Vero cells in 6-well dishes were transfected with pcZF. Cells were also co-transfected with plasmid expressing wild-type p53 or its deletion mutants. Twenty-four hr after transfection, cells were washed with cold PBS and lysed in 250µl/well cold lysis buffer (50mM Tris, pH7.5, 150mM NaCl, 1mM EDTA and 0.1% TritonX-100) containing a protease inhibitor cocktail (Sigma, P8340). After centrifugation at 13,000 xg at 4 ^{o}C, 20µl of cell lysate supernatant were frozen as a pre-immunoprecipitated sample, and a mouse anti-flag antibody (5µg, Sigma, F4042) was added to the remaining supernatant (230µl). The sample was incubated for 12hr with constant gentle agitation. Protein A/G agarose beads (100µl, Pierce, Fisher Scientific, 20421) were added and the samples were incubated for an additional 4hr at 4 ^{o}C. Agarose beads were collected by centrifugation at 13,000 xg at 4 ^{o}C and washed 4 times in a lysis buffer before boiling in an SDS-PAGE sample buffer. Proteins in samples of the unfractionated cell lysate or immunoprecipitates were separated by SDS-PAGE, transferred to membranes and probed with rabbit anti-Zhangfei antisera or rabbit anti-flag (Sigma, F7025). Antibodies were visualized after incubation with Alexa488-labelled anti-rabbit antibody.

5.3.5 Antibodies and immunofluorescence

The antibodies used were rabbit anti-Zhangfei serum (Lu and Misra 2000b), mouse anti-flag antibody (Sigma, F4042), rabbit anti-flag (Sigma, F7025) and mouse anti-GAPDH (Sigma, G8795-200UL). Secondary antibodies were goat anti-mouse Alexa488

(Invitrogen, A-11001), goat anti-rabbit Alexa546 (Invitrogen, A-11035) and goat anti-rabbit Cy5 (Invitrogen, A-10523). Cells were processed for immunofluorescence as described previously (Bergeron et al. 2013). Images were captured using a digital camera attached to an Olympus BX51 microscope and DPmanager software. Captured images were processed using Adobe Photoshop and Illustrator CS6 software.

5.4 Results

5.4.1 p53 forms a complex with Zhangfei via its N-terminal transactivation domain (NTD)

To map the region(s) of p53 required for complex formation with Zhangfei, we assessed the ability of various p53 domains to bind to Zhangfei. FLAG-labeled p53 deletion mutants as well as wild-type p53 (Fig 5.1A) were inserted into expression plasmids (pcDNA3), and were co-expressed with plasmid expressing Zhangfei (pcZF) in Vero cells by transfection. 24h after transfection, cell lysates were prepared and immunoprecipitated with an anti-flag antibody followed by immunoblotting with an anti-Zhangfei antibody. As shown in Fig. 1B, wild-type p53 and some p53 deletion mutants (mutant lanes 2, 3 and 6) co-precipitated with Zhangfei, but other p53 deletion mutants (mutant lanes 4 and 5) were not. These results suggested that only 92 amino acid residues in the N-terminal of p53 (the N-terminal transactivation domain (NTD), which comprises two parts: the transactivation domain (TAD) and the proline rich region (PRR)) were sufficient and required for the interaction with Zhangfei (Fig 5.1B lane 6), while those p53 mutants lacking these regions (Fig 5.1B lane 4) could not bind to Zhangfei.

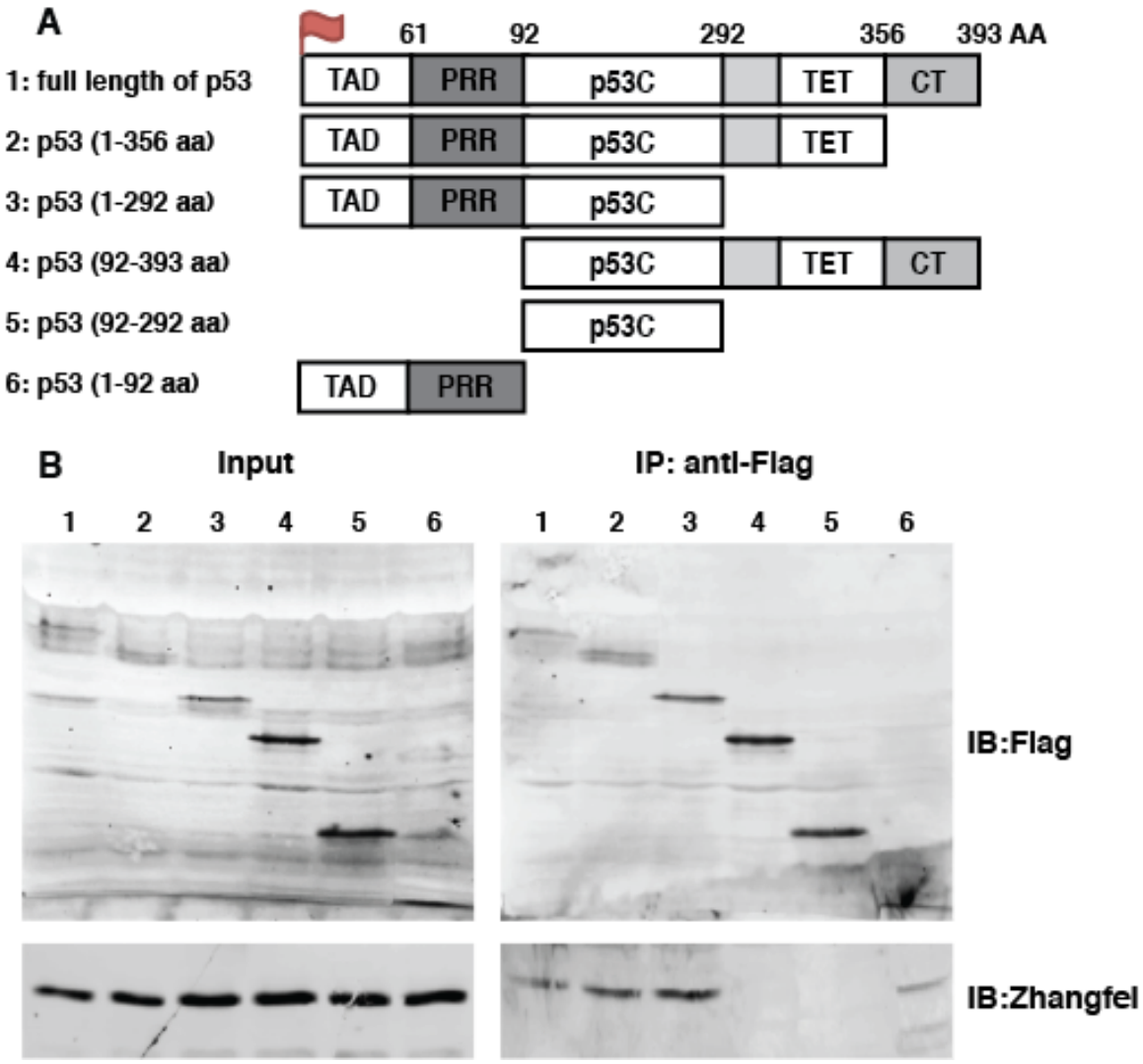

Figure 5.1 Complex formation between Zhangfei and p53. (A) Schematic drawing of the structures of wild-type p53 and its deletion mutants. TAD, transactivation domain; PRR, proline rich region; p53C, central DNA-binding domain; TET, oligomerization domain; CT, basic C-terminal domain. The flag represents the FLAG tag. (B) p53 interacts with Zhangfei through its 92 amino acid residues of N-teminal. Cell lysate from Vero cells previously co-transfected with 1μg of plasmid expressing Zhangfei (pcZF) and 1 μg of plasmid expressing p53 (or its deletion mutants) were incubated with 2 μg of mouse anti-FLAG antibody for 12 hr at 4^{o}C, followed by an additional 4 hr of incubation with 100 μl of Protein A/G agarose beads (IP). The associated proteins were detected by immune-blotting (IB) using an rabbit anti-Zhangfei antibody and a rabbit anti-FLAG antibody.

5.4.2 N-terminal transactivation domain (NTD) is required for Zhangfei-mediated nuclear retention of p53

In previous studies (Chapter 4), we reported that Zhangfei stabilized p53 and promoted its nuclear retention by displacing the E3 ubiquitin ligase mdm2. Mdm2 is a negative regulator of p53, which binds to the NTD domain of p53 and further translocates it out of the nucleus for degradation (Moll and Petrenko 2003). Therefore, we supposed that NTD is also required for the Zhangfei-mediated nuclear retention of p53. To test this hypothesis, we examined the co-localization of Zhangfei and p53 deletion mutants (Fig 5.2 A) by immnofluorescence assay. Consistent with our previous observations (Chapter 4), the expression of Zhangfei enhanced the nuclear localization of wild-type p53 (Fig 5.2B). Similarly, the immunofluorescence showed that Zhangfei expression resulted in an increased nuclear staining of the p53 mutant that only contained the NTD region (1-92 aa), whereas it had no effect on the localization of the fragment containing the remainder of p53 (92-393 aa) (Fig 5.2B). Because this p53 mutant (92-393 aa) was short of the mdm2-binding domain, it was not transported out of the nucleus, and was localized in the nucleus in both the absence and presence of Zhangfei. These results further suggest that the Zhangfei-binding domain in p53 is also the mdm2-binding domain, and Zhangfei achieves its functions through replacing mdm2 and interacting with the NTD region of p53.

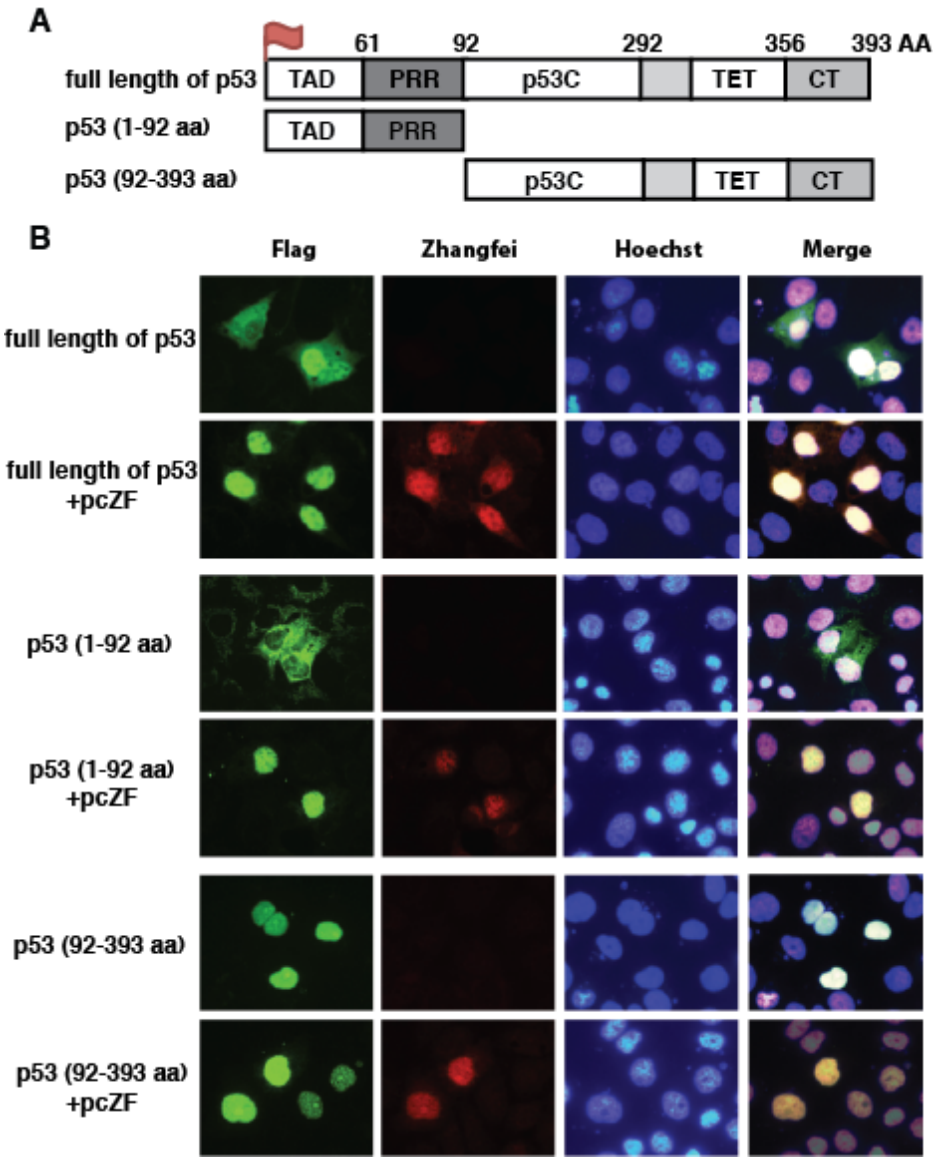

Figure 5.2 Co-localization of Zhangfei and p53 (or its deletion mutants). (A) Schematic drawing of the structures of wild-type p53 and its deletion mutants. TAD, transactivation domain; PRR, proline rich region; p53C, central DNA-binding domain; TET, oligomerization domain; CT, basic C-terminal domain. The flag represents the FLAG tag. (B) Vero cells were transfected with 1 μg of pcZF or a control (pcDNA3). Cells were also co-transfected with a plasmid expressing wild-type p53 or its deletion mutants. 24 h after transfection, p53 as well as Zhangfei were visualized by immunofluorescence with anti-FLAG and anti-ZF antibody. The nucleus was stained with Hoechst dye. Zhangfei alters the subcellular localization of wild-type p53 and the p53 mutant that only contains 92 amino acids in the N-terminus.

5.4.3 Zhangfei enhances p53-mediated transactivation through the N-terminal transactivation domain (NTD) of p53

We then asked whether the NTD region was also required for the effect of Zhangfei on p53-mediated transcriptional activation. To this end, we performed a CAT reporter assay to assess the ability of Zhangfei to indirectly induce transcription from a promoter containing two copies of p53 responsive elements (pCAT3B-p53RE) in the presence of p53 deletion mutants or wild-type p53. As shown in Fig 5.3, Zhangfei enhanced the expression of a reporter gene, CAT, linked to the promoter in the cells expressing wild-type p53 or several p53 mutants that contain NTD region, like p53 (1-356 aa), p53 (1-292 aa) and p53 (1-92 aa), but had no significant effect on the transcription activation of the p53 mutant (92-393 aa). Also, due to the deletion of the transactivation domain, the p53 mutant (92-393 aa) itself didn't activate transcription of its downstream genes.

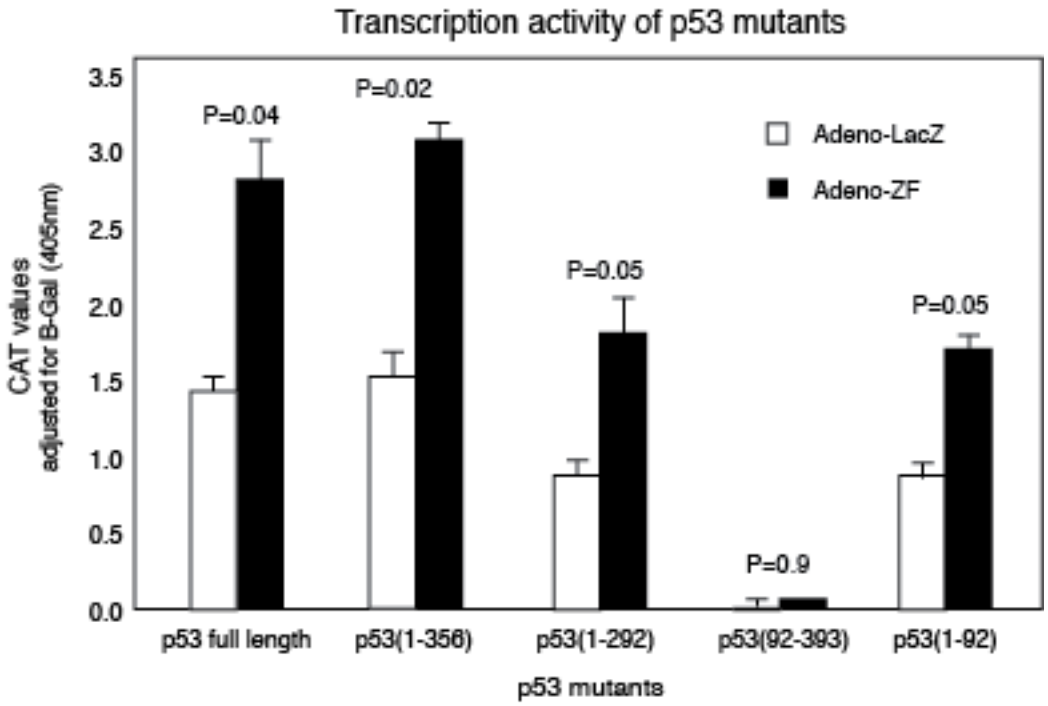

Figure 5.3 Zhangfei activates p53-dependent transactivation via interaction with its 92 amino acids in N-terminal. p53-null MG63 cells were transfected with a reporter plasmid containing the coding sequence for CAT linked to a promoter with two copies of a p53 responsive element (pCAT3B-p53RE, 0.5μg) in the presence or absence of a plasmid expressing Zhangfei (pcZF, 1μg). All samples also contained, as a control, a plasmid expressing b-galactosidase. 24h after transfection, CAT activity was determined. Values represented the relative CAT activity (normalized to the internal control, b-galactosidase) of different treatments. Each datum point is the average of duplicate transfections with the bar representing the range. Standard deviations from means of three independent experiments are shown, and the significant *P* values from t-tests were noted above the bars.

5.5 Discussion

Inactivation of the p53 gene in cancer cells is usually mediated through mutations, direct interaction and transcriptional repression. Mdm2 acts as an E3 ubiquitin protein ligase for p53 and binds to amino acids Phe^{19}, Trp^{23} and Leu^{26} in the N-terminal transactivation domain (NTD) of p53; consequently, this suppresses its transcriptional and pro-apoptotic activity (Momand et al. 1992; Moll and Petrenko 2003). Therefore, the elimination of the negative effect of mdm2 on p53 has been wildly considered to effectively induce apoptosis of cancer cells. According to our previous results (Chapter 4), transcription factor Zhangfei, also known as cAMP Response Element Binding Protein ZF (CREBZF), was a profound positive regulator of p53, which induced the growth arrest and apoptosis of cancer cells by displacing mdm2 and stabilizing p53 (Zhang and Misra 2014). Here, we found that Zhangfei enhanced nuclear retention and increased transcriptional activity of p53 by binding to its N-terminal transactivation domain, providing supportive evidence for our previous conclusions (Zhang and Misra 2014).

In addition to Zhangfei, several other p53 N-terminal binding proteins have been shown to repress or activate the transcriptional activity and stabilization of p53 by masking or uncovering the NTD of p53. These include the p53 negative regulator hepatitis B virus X protein (HBX) (Lee and Rho 2000) and p53 transcriptional coactivators (p300/CBP) (Teufel et al., 2007). HBX represses the transcription of the p53 gene through the E-box element and simultaneously interacts with the p53 protein through the NTD, consequently inhibiting its functions (Wang et al. 1994; Lee and Rho 2000). Like Zhangfei, the transcriptional coactivator p300 and the CREB-binding protein (CBP) interact with N-terminal transactivation domain of p53 and may compete with mdm2 for binding to p53 (Teufel et al. 2007). But, unlike the very tight binding between Zhangfei and p53 (shown in Fig 4.7, Chapter 4), p300/CBP could be outcompeted by other high-affinity p53 N-terminal binding proteins, such as mdm2, leading to the formation of mixed complexes consisting of p53, p300 and mdm2 (Kobet et al. 2000; Teufel et al. 2007). Moreover, mdm2 has been reported to inhibit p300-mediated p53 activation by forming a ternary complex with the two proteins (Kobet et al. 2000).

The competition between Zhangfei and mdm2 for binding to p53 depends on their affinity to the NTD of p53. The mdm2-binding residues of p53 have been identified at 19, 23, and 26 amino acid in the N-terminal (Moll and Petrenko 2003). The occupation of these sites by mdm2 covers the phosphorylation sites of p53, such as Ser15, Thr18 or Ser20, and prevents its activation. To replace mdm2 from p53, Zhangfei may also bind to the same sites with mdm2, or occupy more amino acid residues in the NTD for a higher affinity. Thus, the direct-site mutagenesis of p53 will explore these in future studies.

6. The effect of Zhangfei/CREBZF on cell growth, differentiation, apoptosis, migration, and the UPR in several canine osteosarcoma cell lines

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The purpose of this Chapter was to determine if the effects of Zhangfei on D-17 cells in Chapter 2, 3 and 4 applied more universally to canine OS. I examined three other independently isolated canine OS cell lines—Abrams, McKinley and Gracie, and found that, like D-17, the three cell lines expressed p53 proteins that were capable of activating promoters with p53 response elements on their own, and synergistically with Zhangfei. Furthermore, as with D-17 cells, Zhangfei suppressed the growth and UPR-related transcripts in the OS cell lines. Zhangfei also induced the activation of osteocalcin expression, a marker of osteoblast differentiation and triggered programmed cell death. This Chapter will be submitted for publication as "The effect of Zhangfei/CREBZF on cell growth, differentiation, apoptosis, migration, and the UPR in several canine osteosarcoma cell lines."

My contributions to this Chapter include: I designed and performed all the experiments mentioned in this manuscript. I generated all the final figures and wrote the first draft, which was revised by Professor Vikram Misra. The canine Abrams, Mckinley and Gracie cell lines were kindly provided by Dr. Douglas H. Thamm (Associate Professor of Oncology, Animal Cancer Center, Colorado State University).

6.1 Abstract

We have shown that the bLZip domain-containing transcription factor, Zhangfei/CREBZF inhibits the growth and the unfolded protein response (UPR) in cells of the D-17 canine osteosarcoma (OS) line (Chapter 2) and that the effects of Zhangfei are mediated by it stabilizing the tumour suppressor protein p53 (Chapter 4). To determine if our observations with D-17 cells applied more universally to canine OS, we examined three other independently isolated canine OS cell lines—Abrams, McKinley and Gracie. Like D-17, the three cell lines expressed p53 proteins that were capable of activating promoters with p53 response elements on their own, and synergistically with Zhangfei. Furthermore, as with D-17 cells, Zhangfei suppressed the growth and UPR-related transcripts in the OS cell lines. Zhangfei also induced the activation of osteocalcin expression, a marker of osteoblast differentiation and triggered programmed cell death.

6.2 Introduction

Osteosarcoma (OS) is the most common primary malignant bone tumour in children and adolescents, although its incidence in dogs is ten times greater than in humans (Withrow 2003). Spontaneously occurring osteosarcomas in dogs are an ideal model for cancer research due to their anatomical and physiological similarities with human counterparts (reviewed by (Mueller et al. 2007; Paoloni and Khanna 2008; Khanna et al. 2009).

We have shown that the bLZip domain-containing transcription factor, Zhangfei/CREBZF/SMILE inhibits the growth and the unfolded protein response (UPR) in the D-17 canine osteosarcoma (OS) cell line (Chapter 2) and that the effects of Zhangfei are mediated by stabilizing the tumour suppressor protein p53 (Chapter 4). To determine if our observations with D-17 cells applied more universally to canine OS, we examined three other independently isolated canine OS cell lines—Abrams, McKinley and Gracie. The purpose of this study was to determine the inhibitory role of Zhangfei in these OS cell lines by exploring its potential involvement in growth, differentiation, apoptosis, and metastasis.

Zhangfei was initially identified through its interaction with the host cell factor (HCF1) a protein required for the initiation of herpes simplex virus gene expression (Lu and Misra 2000b). Unlike other bLZip transcription factors, Zhangfei appears to be incapable of binding to consensus bLZip response elements as a homodimer (Cockram et al. 2006). Instead, it fulfills its role in transcriptional regulation by hetero-dimerizing with and modulating other transcription factors or signaling molecules, such as Luman/CREB3 (Misra et al. 2005), Xbp1 (Zhang et al. 2013), ATF4 (Hogan et al. 2006), SMAD 1,5,8 (Lee et al. 2012a), herpes simplex virus VP16 (Akhova et al. 2005), and p53 (Lopez-Mateo et al. 2012).

6.3 Materials and Methods

6.3.1 Cells and tissue culture

Canine osteosarcoma D-17 cells, obtained from the American Type Tissue Culture Collection, were grown in MEM-Alpha containing 10% fetal bovine serum (FBS). Canine Abrams, Mckinley and Gracie cell lines were obtained from Dr. Douglas H. Thamm (Associate Professor of Oncology, Animal Cancer Center, Colorado State University), and grown in Dulbecco's minimal essential medium containing penicillin, streptomycin and 10% newborn calf serum. All media, serum and antibiotics were purchased from Invitrogen (Carlsbad, California).

6.3.2 Adenovirus Vectors Expressing Zhangfei and β-galactosidase (LacZ)

Adenovirus Vectors Expressing Zhangfei and β-galactosidase (LacZ) were constructed, grown, and purified using the Adeno-X Expression System (Clontech). They were created in our laboratory as described earlier (Misra et al. 2005). Cells were infected with Adeno-Zhangfei, Adeno-LacZ (expressing *E. coli* b-galactosidase, LacZ) or mock-infected. A multiplicity of infection (MOI) of 100 plaque-forming units (pfu) per cell was used.

6.3.3 WST-1 Cell Proliferation and Viability Assay

To determine the growth rate of cells, 10^4 cells/well were seeded into 96-well plates. 24h later cells were either mock infected or infected with adenovirus vectors expressing Zhangfei (Adeno-ZF) or b-galactosidase (Adeno-LacZ). Cell proliferation was assessed using Cell Proliferation Reagent WST-1 (Roche, Mannheim, Germany) according to the manufacturer's specifications.

6.3.4 Annex V-apoptosis assay

Cells were collected after trypsinization and stained with Annexin V and propidium iodide using an Annexin V kit (Calbiochem) following manufacturer's instructions. As a positive control cells were treated with 50 μM etopocide (Calbiochem) for 24 hr. Cells were analyzed in a Coulter EPICS XL flow cytometer.

6.3.5 Scratch wound healing assay

Scratch wounds more than 5mm in length and of equal thickness were made in 100% confluent cultures of D-17 or Abrams cells mock-infected or infected with Adeno-ZF or Adeno-LacZ with a 10 µl disposable eppendorf tip. Phase contrast images were taken at 0, 4, 8, 12, and 24 hours after infection from identical regions. The wound size at each time point after infection relative to the starting wound size was measured using Photoshop software in three independent experiments.

6.3.6 Quantitative real-time PCR (qPCR)

Total RNA was extracted using RNeasy Plus Mini Kit from Qiagen (Mississauga, ON, Canada). Gene expression was analyzed by RT-PCR using Brilliant II SYBR Green QPCR Master Mix Kit (Agilent Technologies). The primers used were: Xbp1 spliced-forward: 5'-TCTGCTGAGTCCGCAGCAGG-3', Xbp1 5'-spliced-reverse: TAAGGAACTGGGTCCTTCT-3', HERP-forward: 5'-CCGAGCCTGAGCCCGTCACG -3', HERP-reverse: 5'-CTTTGGAAGCAAGTCCTTGA-3', CHOP-forward: 5'-TGGAAGCCTGGTATGAGGAC-3', CHOP-reverse: 5'-TGCCACTTTCCTCTCGTTC T-3', GRP78-forward: 5'-GGCTTGGATAAGAGGGAAGG-3', GRP78-reverse: 5'-GGTAGAACGGAACAGGTCCA-3', osteocalcin-forward: 5'-AAGCRGGAGGGCAGC AGGT-3', osteocalcin-reverse: 5'-CYGRTARGCYTCCTGRAAGC-3'.

6.3.7 PCR and sequencing of p53 genes

The sequences of PCR primers used for canine p53 amplification were: canine p53-forward: GGTGACTGCAATGGAGGAGTCGCA, canine p53-reverse: TCAGTCTGAGTCAAGCCCTTCTCT. RNA was purified from cells using the RNEasy Plus mini kit with a genomic DNA elimination step (Qiagen) and RNA converted to cDNA with the Quantitect Reverse Transcription kit (Qiagen) using instructions supplied by the manufacturer. Two-step RT-PCR reactions used TopTaq emzyme (Qiagen) and were performed in a PCR machine.

6.3.8 Plasmids and chloramphenicol acetyl transferase (CAT) assay

The construction of pcZF (Lu and Misra, 2000), a plasmid that expresses Zhangfei in mammalian cells, has been described. The CAT reporter plasmid pCAT3B-p53RE was constructed by transferring oligonucleotides containing two copies of p53 responsive element, GGTCAAGTTGGGACACGTCCaaGAGCTAAGTCCTGACATGTCT (IDT, Coralville, Iowa), to pCAT3Basic (Promega), which contains the coding sequence for CAT linked to a basal promoter. Oligonucleotides representing the p53 responsive elements with overhanging 5' terminal KpnI and 3' terminal BglII sites were annealed and ligated to pCAT3Basic cut with the same enzymes.

In CAT assay, D-17, Abrams, Gracie and McKinley cells were transfected with 0.5μg of pCAT3B-p53RE, in the presence or absence of a plasmid expressing Zhangfei (pcZF, 1μg), using Lipofectamine 2000 (Invitrogen) as described in the manufacturer's instructions. The promoter-less parental reporter plasmid, pCAT3B was included as a control to show basal CAT activity. 250 ng of pCMVBGal, a plasmid specifying β-galactosidase, were added to each transfection as an internal control. 24h after transfection, the CAT activity was determined by ELISA. CAT values were normalized to β-galactosidase.

6.3.9 Antibodies, immunoblotting and immunofluorescence

The antibodies used were mouse anti-FLAG (Sigma), rabbit anti-Zhangfei serum, rabbit anti-Xbp1 (Abcam, Cambridge, MA), rabbit anti-HERP (Abcam, Cambridge, MA), rabbit anti-GRP78 (Abcam, Cambridge, MA), and mouse anti-GAPDH (Chemicon, Billerica, MA). Secondary antibodies were goat anti-mouse Alexa488, goat anti-rabbit Alexa546 and goat anti-rabbit Cy5 (Invitrogen). Cells were processed for immunoblotting and immunofluorescence as described previously (Lu and Misra 2000b; Bergeron et al. 2013).

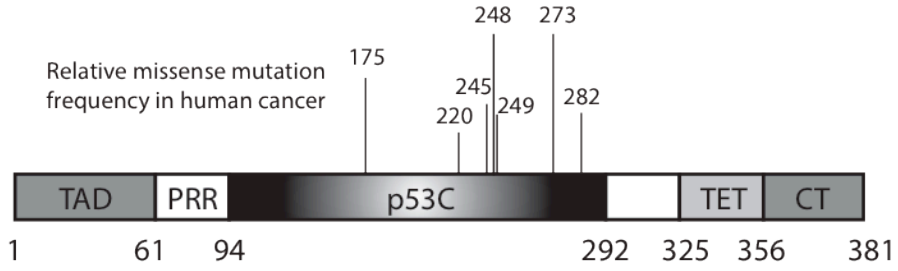
6.4 Results

6.4.1 All four canine OS cells lines express functional p53

To determine the effects of Zhangfei we had observed in D-17 OS cells we examined three other canine OS cell lines. We have shown that Zhangfei exerts its effect on cell growth and the UPR by stabilizing p53 (Chapter 4, Zhang and Misra, 2014) and it therefore has no effect on cancer cells that do not possess functional p53. To assess the status of p53 in the canine cell lines I amplified p53 transcripts from the cells using PCR and determined the nucleotide sequences of the products. Figure 6.1B shows the derived amino acid sequences of p53 from the cell lines and the reference sequence from the canine genome database. All four cell lines contained transcripts for p53 that, with the exception of a few amino acid variations, were identical to the reference sequence. None of the amino acid polymorphisms in the sequences were at positions identified as important for p53 function (Joerger and Fersht 2008)(Fig 6.1A).

To determine if the p53 proteins in the cell lines were functionally active, I transfected the cells with a plasmid that expressed the reporter protein chloramphenicol acetyl transferase (CAT) regulated by a promoter with two copies of a p53 response element (pCATp53RE). As a negative control, cells were transfected with a plasmid (pCAT3B) without the response elements. Parallel cultures were transfected with a plasmid expressing Zhangfei. Figure 6.1C shows that expression of CAT was activated in all four cell lines in a p53 response element–dependent manner and that the presence of Zhangfei enhanced expression.

A



B

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D-17             1 MEESQSELNIDPPLSQETFSELWNLLPENNVLSSELCPAVDELLLPESVVNWLDEDSDDA  60
Abrams           1 MEESQSELNIDPPLSQETFSELWNLLPENNVLSSELCPAVDELLLPESVVNWLDEDSDDA  60
Gracie           1 MEESQSELNIDPPLSQETFSELWNLLPENNVLSSELCPAVDELLLPESVVNWLDEDSDDA  60
Mckinley         1 MEESQSELNIDPPLSQETFSELWNLHPENNVLSSELCPAVDELLLPESVVNWLDEDSDDA  60
                   ************************* ********************************

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D-17            61 PRMPATSAPTAPGLAPSWPLSSSVPSPKTYPGTYGFRLGFLHSGTAKSVTWTYSPLLNKL 120
Abrams          61 PRMPATSAPTAPGPAPSWPLSSSVPSPKTYPGTYGFRLGFLHSGTAKSVTWTYSPLLNKL 120
Gracie          61 PRMPATSAPTAPGPAPSWPLSSSVPSPKTYPGTYGFRLGFLHSGTAKSVTWTYSPLLNKL 120
Mckinley        61 PRMPATSAPTAPGPAPSWPLSSSVPSPKTYPGTYGFRLGFLHSGTAKSVTWTYSPLLNKL 120
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                                                                        175
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D-17           121 FCQLAKTCPVQLWVSSPPPPNTCVRAMAIYKKSEFVTEVVRRCPHHERCSDSSDGLAPPQ 180
Abrams         121 FCQLAKTCPVQLWVSSPPPPNTCVRAMAIYKKSEFVTEVVRRCPHHERCSDSSDGLAPPQ 180
Gracie         121 FCQLAKTCAVQLWVSSPPPPNTCVRAMAIYKKSEFVTEVVRCCPHHERCSDSSDGLAPPQ 180
Mckinley       121 FCQLAKTCPVQLWVSSPPPPNTCVRAMAIYKKSEFVTEVVRRCPHHERCSDSSDGLAPPQ 180
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                                                  220
Canine WT p53  181 HLIRVEGNLRAKYLDDRNTFRHSVVVPYEPPEVGSDYTTIHYNYMCNSSCMGGMNRRPIL 240
D-17           181 HLIRVEGNLRAKYLDDRNTFRHSVVVPYEPPEVGSDYTTIHYNYMCNSSCMGGMNRRPIL 240
Abrams         181 HLIRVEGNLRAKYLDDRNTFRHSVVVPYEPPEVGSDYTTIHYNYMCNSSCMGGMNRRPIL 240
Gracie         181 HLIRVEGNLRAKYLDDRNTFRHSVVVPYEPPEVGSDYTTIHYNYMCNSSCMGGMNQRPIL 240
Mckinley       181 HLIRVEGNLRAKYLDDRNTFRHSVVVPYEPPEVGSDYTTIHYNYMCNSSCMGGMNRRPIL 240
                   ************************************************************.****
                      245 248-9                    273      282
Canine WT p53  241 TIITLEDSSGNVLGRNSFEVRVCACPGRDRRTEEENFHKKGEPCPEPPPGSTKRALPPST 300
D-17           241 TIITLEDSSGNVLGRNSFEVRVCACPGRDRRTEEENFHKKGEPCPEPPPGSTKRALPPST 300
Abrams         241 TIITLEDSSGNVLGRNSFEVRVCAFPGRDRRTEEENFHKKGEPCPEPPPGSTKRALPPST 300
Gracie         241 TIITLEDSSGNVLGRNSFEVRVCACPGRDRRTEEENFHKKGEPCPEPPPGSTKRALPPST 300
Mckinley       241 TIITLEDSSGNVLGRNSFEVRVCACPGRDRRTEEENFHKKGEPCPEPPPGSTKRALPPST 300
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D-17           301 SSSPPQKKKPLDGEYFTLQIRGRERYEMFRNLNEALELKDAQSGKEPGGSRAHSSHLKAK 360
Abrams         301 SSSPPQKKKPLDGEYFTLQIRGRERYEMFRNLNEALDLKDAQSGKEPGGSRAHSSHLKAK 360
Gracie         301 SSSPPQKKKPLDGEYFTLQIRGRERYEMFRNLNEALELKDAQSGKEPGGSRAHSSHLKAK 360
Mckinley       301 SSSPPQKKKPLDGEYFTLQIRGRERYEMFRNLNEALELKDAQSGKEPGGSRAHSSHLKAK 360
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Canine WT p53  361 KGQSTSRHKKLMFKREGLDSD 381
D-17           361 KGQSTSRHKKLMFKREGLDSD 381
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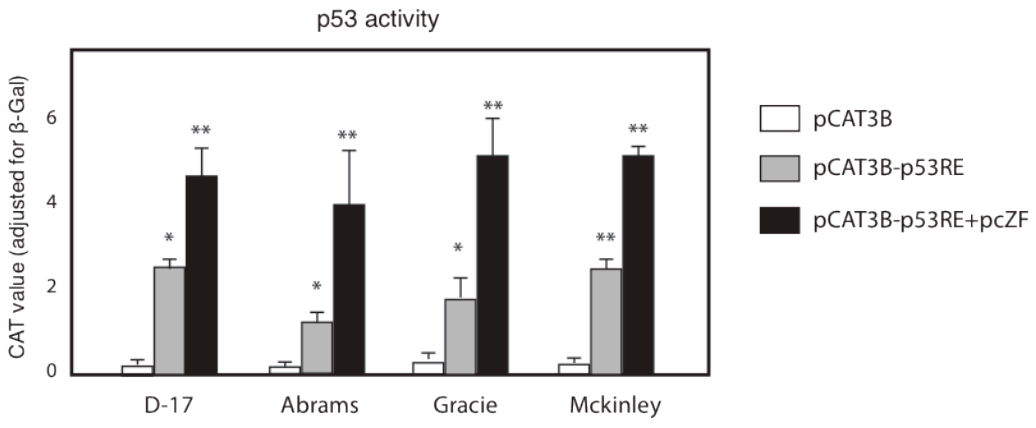

Figure 6.1. p53 in dog osteosarcoma cell lines. (A) Schematic structure of full-length p53. TAD: N-terminal transactivation domain; PRR: proline-rich region; p53C: central DNA-binding domain; TET: tetramerization domain; CT: extreme C terminus. p53C is the domain where most cancer-associated p53 mutations are located. The numbers above the diagram represent the residues with highest frequency of oncogenic missense mutations (Joerger and Fersht 2008). (B) Derived amino acid sequence alignment of p53s from 4 dog osteosarcoma cell lines and dog wild-type p53. The amino acid sequences were aligned using MacVector software. The residues that have high mutant frequency were marked above the diagram. (C) p53s of dog osteosarcoma cells have transcription activity, and Zhangfei enhances p53-dependent transactivation. D-17, Abrams, McKinley, and Gracie cells were transfected with 0.5μg of pCAT3B or pCAT3B-p53RE, in the presence or absence of 1μg of pcZF. 24h after transfection, the CAT activity was determined. Values represented the relative CAT activity (adjusted by β-galactosidase) of different treatments. Standard deviations from means of three individual experiments are shown (*P<0.05, **P<0.01).

6.4.2 Cellular outcome following ectopic expression of Zhangfei: growth cessation, apoptosis and differentiation

We next compared the effect of Zhangfei on the growth characteristics of Abrams, Mckinley, and Gracie cells with its effect on D-17 cells. The cells were infected with adenovirus expressing either Zhangfei (Adeno-ZF) or the control protein b-galactosidase (Adeno-LacZ). Cell growth was monitored by the WST-1 Cell Proliferation Assay. In agreement with previous results, all four Adeno-ZF-infected cells failed to divide as early as day 1 after infection as determined by their ability to convert WST-1 Cell Proliferation reagent and absorb light at 405 nm. Mock infected cells continued to grow for three days and the growth of Adeno-LacZ-infected cells was indistinguishable from mock-infected cells (Fig 6.2).

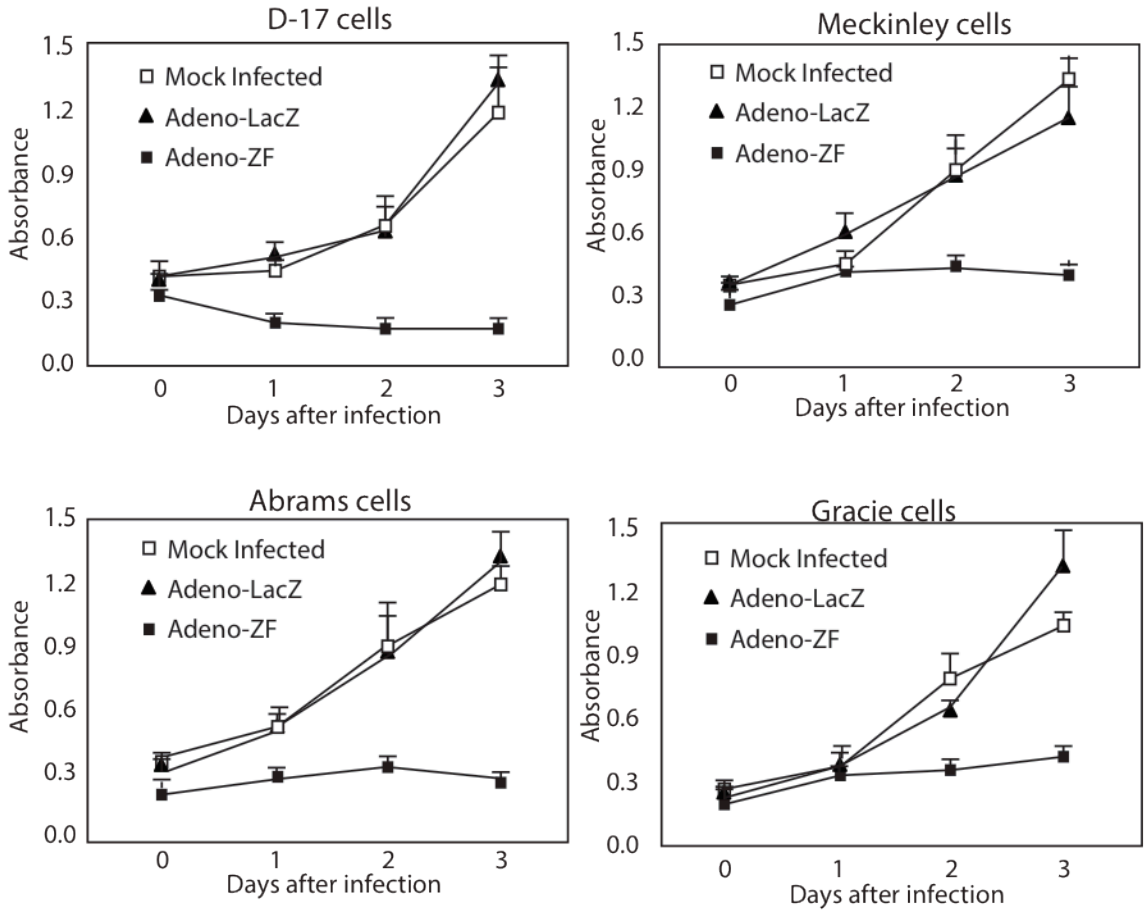

Figure 6.2. Ectopic expression of Zhangfei suppresses cell growth in canine osteosarcomas. D-17, Abrams, McKinley, and Gracie canine OS cells were mock-infected or infected with adenovirus vectors expressing either Zhangfei (Adeno-ZF) or beta-galactosidase (Adeno-LacZ) and measured growth rates by absorbance at 405 nm with WST-1 at different time points after infection.

Since Zhangfei may stop cell growth by inducing differentiation and/or causing apoptosis, we further performed a transcript level analysis of the osteosarcoma differentiation marker—osteocalcin (Ciovacco et al. 2009) and a flow cytometric analysis of Annexin V-stained apoptotic cells in D-17 and Abrams cells infected with either Adeno-ZF or Adeno-LacZ. Compared with LacZ-expressing and even vitamin D3-treated cells (negative and positive controls, respectively), Zhangfei significantly increased the expression of osteocalcin transcripts in a time-dependent manner (Fig 6.3). Meanwhile, we also found both D-17 and Abrams cells began to undergo apoptosis upon expression

of Zhangfei as the percentage of cells with Annexin V stain increased to ~10% by 12h after infection and to >22% by 24h, while the mock infected and LacZ-expressing cells had obviously lower percentages of apoptotic cells (Fig 6.4).

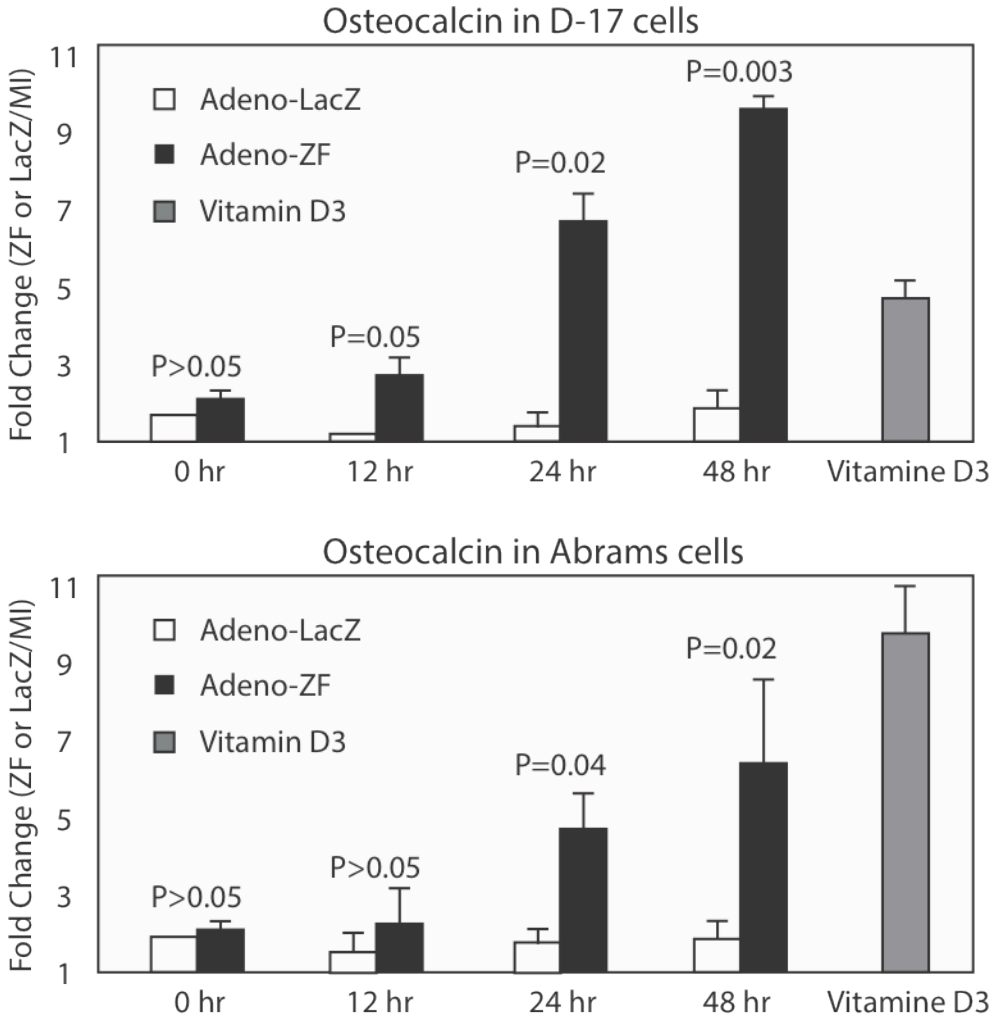

Figure 6.3. Zhangfei induces differentiation of canine osteosarcoma cells. D-17 and Abrams cells were either mock-infected or infected with Adeno-ZF or Adeno-LacZ. The positive control cells were treated with 10^{-5} mM of vitamin D3. The mRNA levels of osteoblast differentiation marker (osteocalcin) were estimated by qRT-PCR.

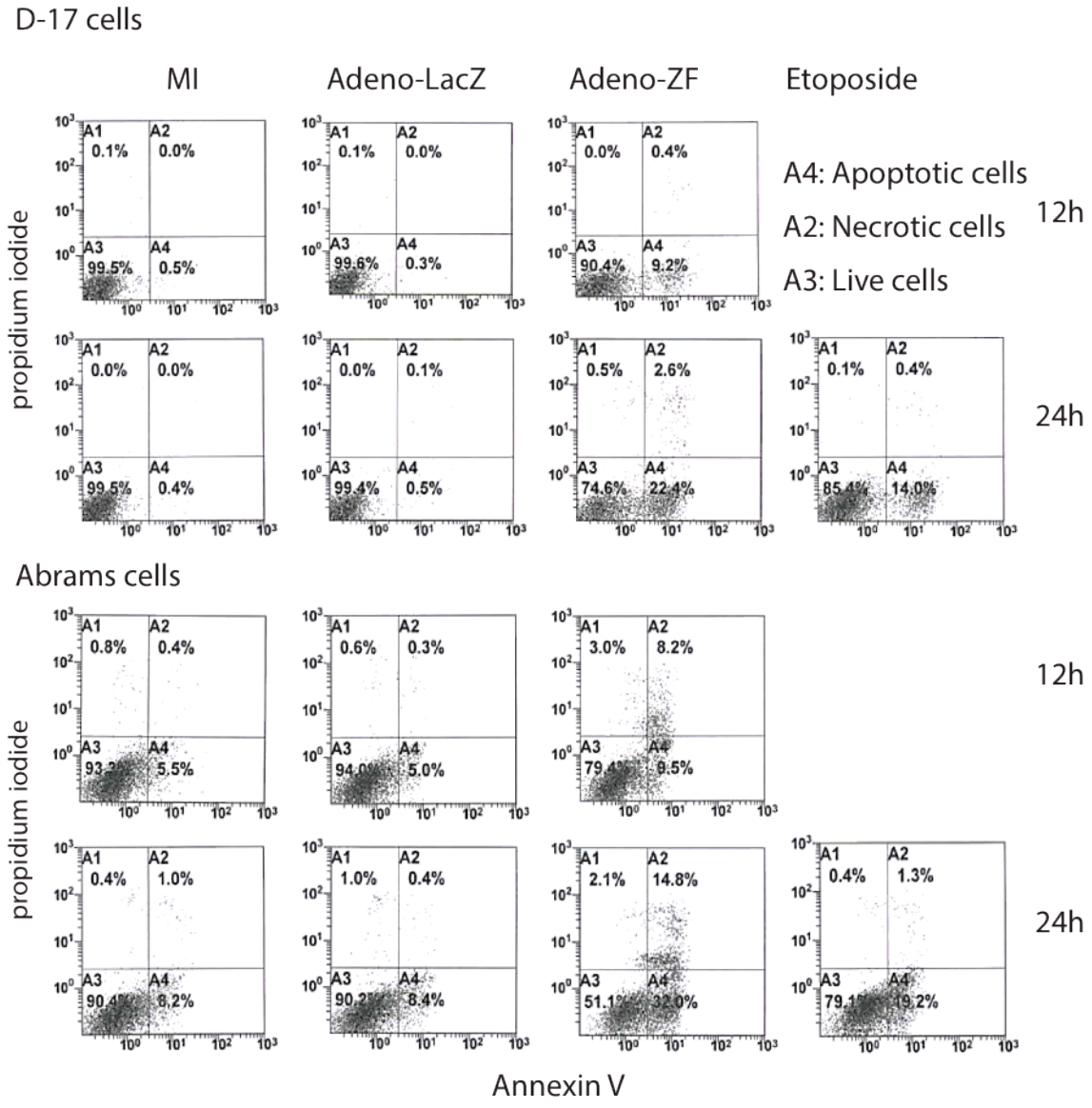

Figure 6.4. Zhangfei causes canine osteosarcoma cells to commit apoptosis. In D-17 and Abrams cells, Adeno-ZF or Adeno-LacZ infected cells, mock-infected cells and cells treated with 50 μM of etoposide (positive control) were stained with Annexin V-fluorescence and propidium iodide, and cells staining with either or both dyes were enumerated by FACS. A4 represents the percentage of total cells undergoing apoptosis.

6.4.3 Expression of Zhangfei suppresses migration of canine osteosarcoma cells

Migratory/metastatic behaviour in cancer cells is a typical hallmark of malignancy. To investigate whether ectopic expression of Zhangfei correlated with altered migratory behaviour, we performed cell motility assays on the canine osteosarcoma cultures. Following scratch wounding, wound closure was significantly slower in cultures (D-17 and Abrams canine cells) infected with Adeno-ZF compared to cultures infected with Adeno-LacZ or mock-infected cells (Fig 6.5), showing that the ectopic expression of Zhangfei indeed causes decreased cell motility in canine osteosarcoma cells.

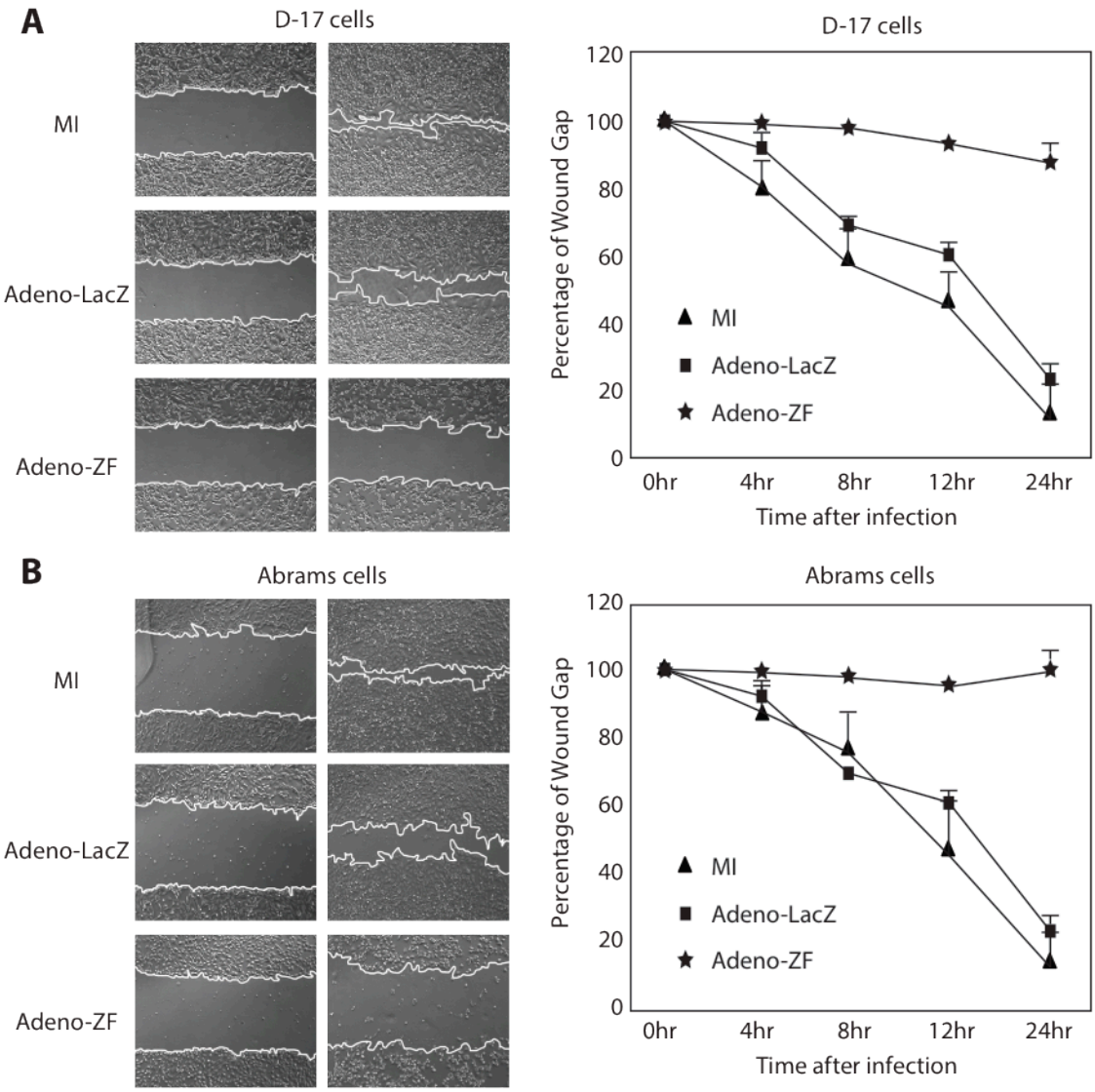

Figure 6.5 Ectopic expression of Zhangfei causes decreased cell motility in canine osteosarcoma cells. (A) Scratch wounds were made in 100% confluent cultures of D-17 or Abrams cells mock-infected or infected with Adeno-ZF or Adeno-LacZ. Phase contrast images were taken at 0, 4, 8, 12, and 24 hours after infection from identical regions. (B) The wound size relative to the starting wound size was measured at each time point after infection in three independent experiments and expressed as a percentage reduction in wound size + standard deviation (s.d.).

6.4.4 Zhangfei negatively regulates the UPR in canine osteosarcomas

The unfolded protein response (UPR) is an adaptive cellular stress response that alleviates ER stress or, failing, induces apoptosis. In previous studies (Chapter 2), we found Zhangfei was a negative regulator of the UPR in D-17 canine osteosarcoma cells (Bergeron et al. 2013). To investigate if Zhangfei could consistently suppress the UPR in other canine osteosarcoma cells, the four canine osteosarcoma cell lines infected with either Adeno-ZF or Adeno-LacZ were treated with the UPR pharmacological inducer thapsigargin, or were deprived of glucose. The latter treatment is a known physiological inducer of the UPR. The levels of UPR transcripts (Xbp1s, HERP, CHOP and GRP78) activated by thapsigargin (Fig 6.6A) and glucose deprivation (Fig 6.6B) were dramatically decreased in Zhangfei-expressing canine cell lines, in contrast, LacZ had no obvious effect on the ability of thapsigargin or glucose deprivation to activate the UPR. In addition, this decrease in mRNA was reflected in a decrease in UPR proteins (Xbp1s, HERP, and GRP78) in thapsigargin-treated D-17 (shown by Chapter 1) and Abrams (Fig 6.6C) cells. Fig 6.6D, which showed intracellular proteins detected by immunofluorescence, also supported these data—the Xbp1s protein was undetectable in D-17 and Abrams cells expressing Zhangfei.

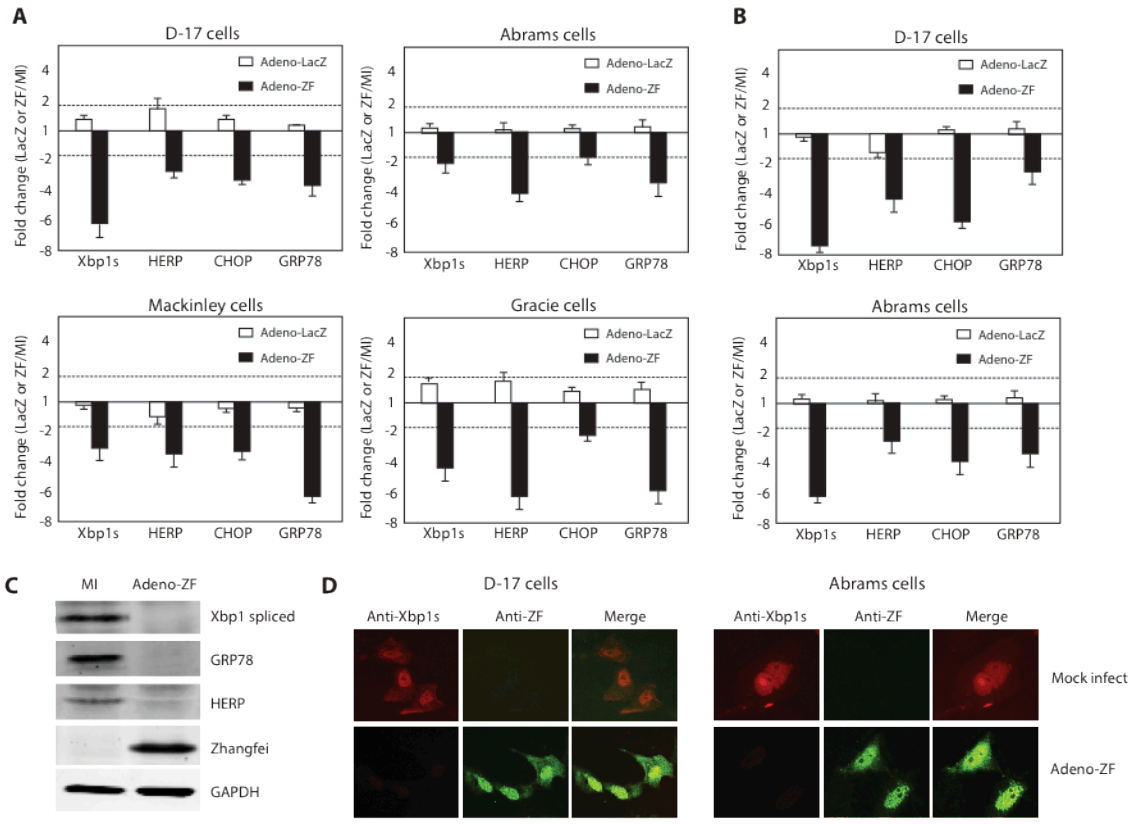

Figure 6.6 Zhangfei negatively regulates the Unfolded Protein Responses (UPR) in canine osteosarcomas. Zhangfei suppresses the mRNA levels of UPR transcripts activated by thapsigargin (A) and glucose deprivation (B). D-17, Abrams, McKinley, and Gracie canine OS cells were either mock-infected or infected with Adeno-ZF or Adeno-LacZ. 24h after infection, cells were treated with thapsigargin (A) for 4h or glucose-free medium (B) for 24h, and then cells were harvested, and differences in mRNA levels of transcripts (Xbp1s, HERP, CHOP, and GRP78) were determined by qRT-PCR (C) and (D). Zhangfei downregulates UPR proteins. D-17 and Abrams cells were either mock-infected or infected with Adeno-ZF. 24h after infection, cells were treated with thapsigargin for 4h, and UPR proteins (spliced Xbp1, HERP, and GRP78) were detected by western blots (C) and immunofluorescence (D).

6.4 Discussion

Canine OS is an aggressive tumour that accounts for approximately 85% of primary bone tumours in dog (Selvarajah and Kirpensteijn 2010). OS causes local skeletal destruction resulting in osteoproductive and osteolytic lesions, and it is highly metastatic to the lungs. Although there has been dramatic progress in the standard treatments of OS, including amputation, chemotherapy, and palliative radiation, these therapies often fail, leading to recurrence of the tumour and metastatic spread (Brodey and Abt 1976; Tomlin et al. 2000; Walter et al. 2005; Fan et al. 2009). Over the years, combined therapies, such as chemotherapy combined with immune modulators, have been practiced on dog osteosarcoma (Dow et al. 2005), although with poor overall survival times.

In previous studies (Chapter 2), we found that the transcription factor CREBZF/Zhangfei suppressed the growth of D-17 dog OS cells (Bergeron et al. 2013). Herein, we further discovered that the growth suppressive effects of Zhangfei were applicable to three other independently isolated canine OS cell lines as well.

The unfolded Protein Response (UPR) is an adaptive response induced by endoplasmic reticulum (ER) stress, which alleviates ER stress by up-regulating the expression of ER-resident chaperons (Kaufman et al. 2002), inducing ER-associated protein degradation (ERAD), and down-regulating the synthesis of new proteins (Meusser et al. 2005; Lai et al. 2007). If these mechanisms are not sufficient to alleviate ER stress, then an apoptosis program is initiated to induce cell death. My results (Chapter 3) (Zhang et al. 2013) suggest that Zhangfei is a potential regulator of the UPR, and it might accelerate UPR feedback mechanisms by interacting with the UPR mediator-Xbp1 and targeting it for proteasomal degradation. In the present study, the strong inhibitory effects of Zhangfei on both pharmacological (thapsigargin) and physiological inducer (glucose deprivation) – induced UPR was also observed in the four canine osteosarcoma cell lines we examined.

Zhangfei suppresses the UPR and cell growth by stabilizing the tumour suppressor protein p53 (Chapter 4). All four canine OS cell lines we examined express functional p53 (Figure 6.1). These results suggest that the induction of Zhangfei expression in canine OS may be an effective strategy for suppressing cell growth and metastasis.

However, the strategy would likely only be successful with OS that had functional p53. A large proportion of human cancers have deleted or otherwise non-functional p53. At present we do not know the proportion of canine OS that have inactive p53. Some studies (Mendoza et al. 1998) suggest that most canine OS do not have deletions or major rearrangements in the gene for p53. Although 30-50% of p53 coding sequences in canine OS have polymorphisms (van Leeuwen et al. 1997; Johnson et al. 1998; Mendoza et al. 1998) the effect of these changes on p53 functionality is unknown. It is therefore difficult to determine how universally applicable Zhangfei would be as a modality for treating canine OS. The role of p53 in canine OS is also controversial. The expression of ectopic p53 in canine OS cells, both *in vitro* and *in vivo* models leads to reduced tumour growth and an increase in apoptotic cells (Yazawa et al. 2003; Kanaya et al. 2011; Bergeron et al. 2013). In contrast, other studies in both humans (Fu et al. 2013) and dogs (Sagartz et al. 1996; Loukopoulos et al. 2003) suggest that increased p53 expression in OS correlates with more aggressive tumours and decreases survival time.

7. General discussion and conclusions

Zhangfei is a cellular transcriptional repressor identified in our laboratory through its interaction with a cellular co-factor (HCF1) that is required for initiating Herpes Simplex Virus-1 gene expression during infection (Lu and Misra 2000b). In the previous studies, we found that Zhangfei was implicated in the differentiation and death of human medulloblastoma cells (Bergeron et al. 2013). The main goal of this study was to further determine the effect of transcription factor Zhangfei on the growth of osteosarcoma cells and the molecular mechanisms responsible. Given the important role of the UPR in maintaining malignancy in cancer as well as the suppressive ability of Zhangfei on many other cellular regulators (Lu and Misra 2000b; Misra et al. 2005; Xie et al. 2008; Misra et al. 2012), we developed techniques to monitor the UPR in dog and human osteosarcoma cells. We tested the hypothesis that the ectopic expression of Zhangfei in these cells would suppress their ability to activate the UPR and, consequently, inhibit cell growth. As described in Chapter 2, we revealed that while Zhangfei inhibited both the UPR pathways and the growth of D-17 dog cells, it had no effect on the UPR and relatively modest, but significant, effect on the growth of Saos-2 human cells. Although these data suggested there might not be a direct link between the UPR suppression and cell growth arrest induced by Zhangfei, it was foundational to the investigation presented in this thesis since it demonstrated the selectively suppressive effects of Zhangfei on both cell growth and the UPR in OS cell lines.

The UPR pathways are mediated by three bLZip-containing transcription factors—Xbp1, ATF4 and ATF6. The similarity of the bLZip domains of these UPR mediators to Zhangfei, suggested the possibility that Zhangfei might associate with them influencing their ability to mediate the UPR. Thus, we hypothesized that Zhangfei may interact with Xbp1, ATF6 and ATF4 by its leucine zipper and prevent these transcription factors from activating UPR genes. As described in Chapter 3, we showed that Zhangfei had a suppressive effect on most UPR genes activated in response to the drug thapsigargin, and this effect was mediated, at least partially, by the leucine zipper dependent interaction of Zhangfei and Xbp1s, which resulted in the subsequent proteasomal degradation of Xbp1s (Zhang et al. 2013). It should be emphasized that while we showed the direct effect of

Zhangfei on Xbp1, Zhangfei may suppress the other UPR-inducing bLZip factors as well. We have previously shown that Zhangfei can suppress the activity of ER-resident bLZip protein Luman/CREB3 but not ATF6 (Lu and Misra 2000b; Misra et al. 2005). Recently, Misra and others (Misra et al. 2012) also showed that Zhangfei/SMILE suppresses the ability of CREBH to induce UPR genes in hepatoma cells. While the findings in Chapter 3 and those of others showed that Zhangfei had the ability to suppress the UPR pathways by interacting with Xbp1 as well as other bLZip proteins, it was still unclear why the effects of Zhangfei were not universal—Zhangfei had no obvious effects on untransformed cells and some cancer cell lines (as showed in Chapter 2). Thus, more detailed understandings about the role of Zhangfei in regulation of both the UPR and cell growth are clearly needed.

Neoplastic transformation occurs when gene mutations in a single cell make it unable to repair DNA damage and trigger programmed cell death. The most common examples of this are the inactivation of tumour suppressor genes and the up-regulation of oncogenes, leading to uncontrolled proliferation of the cells with damaged genetic information. Among the many genetic lesions in cancer, the inactivation of p53 function is the most universal event and more than 50% of human cancers contain p53 mutation that alter or abrogate its tumour suppressor functions (Lane et al. 2010). The high frequency of p53 inactivation in cancer points to its importance in preventing oncogenesis and makes this gene a good target for the development of new cancer therapies. Gene therapy using wild-type p53 has been demonstrated to restore p53 functions and lead to tumour regression in several animal models (Martins et al. 2006; Ventura et al. 2007; Xue et al. 2007). With the growing understanding of the structure and regulation of p53, several gene therapeutic strategies have been employed in the attempt to activate p53 in cancers that still retain a functional protein. For example, treatment of colorectal adenocarcinoma cells that have functional p53 with siRNA against mdm2 inhibited the growth of the cells and rendered them more sensitive to cisplatin (Yu et al. 2006). Crystal structure analysis showed that nutlin, an antagonist of the mdm2-p53 interaction, binds to mdm2 and prevents its interaction with p53. This allows the activation of p53 pathways inducing cell cycle arrest and apoptosis in mice xenograft tumours (Vassilev et al. 2004).

Based on the observations that Zhangfei has profound inhibitory effects on cell growth and the UPR in some cancer cells, but not in normal cells and other tumour cells, I hypothesized that Zhangfei acts through an intermediary that is either not activated or is defective in cells that it does not affect. I also hypothesized that this intermediary was p53. The investigation described in Chapter 4 demonstrated that Zhangfei enhanced the activation of wild-type p53 by interacting with p53 and displacing its negative regulator mdm2 from association with p53, thereby leading to inhibition of the UPR pathways and growth arrest of osteosarcoma cells (Zhang and Misra 2014). Based on the observations that Zhangfei only suppressed the growth and the UPR in wild-type p53-expressing, but not in p53-null human osteosarcoma cells (in Chapter 4), we speculated that the differential influences on the UPR and growth of Saos-2 cells, described in Chapter 2, could be because these cells possess mutated p53 (Smardova et al. 2005), which only partially inhibited its functions. Moreover, these findings are important, not only because they provided a reasonable explanation for the "selective suppression" phenomenon of Zhangfei, but that they demonstrated a novel mechanism to reactivate wild-type p53 in cancer cells and provided a "bigger picture" on the interaction between p53, the UPR and Zhangfei in the regulation of cancer cell growth.

As discussed in Chapter 4, the induction of the UPR is a protective mechanism utilized by neoplastic cells to adapt to ER stress and to promote immune resistance, cancer progression, and drug resistance (Moenner et al. 2007; Ron and Walter 2007). Therefore, in addition to the cell cycle arrest and apoptosis directly induced by the activated p53, the inhibition of the UPR, mediated by p53, represents another potential strategy by which Zhangfei could be used to inhibit cancer. Further, the fact that ER stress, and the resulting activation of the UPR induces p53 cytoplasmic localization and prevents p53-dependent apoptosis (Qu et al. 2004) suggests the existence of an ER stress-p53-UPR regulatory loop (Fig 4.8, Chapter 4). However, I was not able to define the specific mechanisms by which p53 conversely inhibits the UPR pathways. p53 and mdm2 form an autoregulatory negative feedback loop aimed at controlling levels of nuclear p53 (Moll and Petrenko 2003). Mdm2 is an E3 ubiquitin ligase that recongnizes its target protein and mediates its ubiquitylation and degradation. Mdm2 is principal cellular antagonist of p53 and promotes p53 degradation through an ubiquitin-dependent pathway on 26S proteasomes

(Honda et al. 1997); p53, in turn, stimulates the expression of mdm2, and thus operates in a negative feedback loop to prevent the overactivation of p53 (Wu et al. 1993). However, under the stress conditions, mdm2 induced by the activated p53 accumulates in cells but is not able to interact with p53 due to its phosphorylation. Given the previous observations that Zhangfei stabilizes p53 by displacing mdm2 (in Chapter 4), as well as Zhangfei suppresses UPR mediator Xbp1 by targeting it for proteasomal degradation (in Chapter 3), we speculate that Zhangfei and p53 may mediate the inhibition of UPR by stimulating and recruiting mdm2 that subsequently recognizes the UPR-related proteins and target them for degradation. To test this hypothesis further investigations are needed.

Chapter 5 revealed that the N-terminal transactivation domain (NTD) of p53 was responsible for the complex formation with Zhangfei. This work was significant because it further confirmed that the Zhangfei-binding domain of p53 was closely associated with its mdm2-binding domain and transcription activation region. Together with the observations in Chapter 4, therefore, reactivation of p53 by Zhangfei-mediated displacement of mdm2 from the p53-mdm2 complex may represent a novel mechanism for cancer gene therapy.

Taken together, the general conclusions of this thesis are:

1. Zhangfei profoundly inhibits the UPR and cell growth in some tumour cells, but not in normal cells or in some other tumour cells.
2. Zhangfei is a negative regulator of the UPR mediator Xbp1s.
3. Zhangfei is able to activate p53 signaling; consequently, suppresses the UPR pathways and induces cell growth arrest only in the osteosarcoma cells with functional p53.

The specific conclusions of each chapter are:

Chapter 2: Effect of Zhangfei on Canine/Human Osteosarcoma Cells

- Zhangfei dramatically suppresses the growth of canine D-17 osteosarcoma cells. While the effect of Zhangfei on Saos-2 human cells is not as dramatic as on D-17

cells, the growth rate of these cells is significantly suppressed compared with the negative controls.

- Zhangfei induces macropinocytosis and apoptosis in D-17 cells.
- Zhangfei inhibits thapsigargin-induced UPR only in canine D-17 cells, but not in human Saso-2 cells, suggesting that the ability of Zhangfei to suppress the UPR and tumour cells growth may not be linked.

Chapter 3: Zhangfei is a Negative Regulator of the UPR Mediator Xbp1s

- Zhangfei has a suppressive effect on most UPR genes activated by the calcium ionophore thapsigargin.
- Zhangfei suppresses the UPR, at least partially, due to the interaction of Zhangfei with Xbp1s, which leads to the subsequent proteasomal degradation of Xbp1s. The bLZip domain of Zhangfei is required for this interaction.
- Zhangfei suppresses the ability of Xbp1s to activate transcription from a promoter containing unfolded protein response elements and significantly reduces the ability of Xbp1s to activate the UPR.

Chapter 4: Effects of Zhangfei on the UPR and Cell Growth are Exerted Through the Tumour Suppressor p53

- Zhangfei is a positive regulator of p53. In cells ectopically expressing Zhangfei, the protein stabilizes p53 and promotes its nuclear retention.
- Suppression of p53 by siRNA partially restores cell growth and the UPR that are inhibited by Zhangfei, indicating that the inhibitory influences of Zhangfei, at least in part, are mediated by p53.
- Zhangfei interacts with p53 and displaces the E3 ubiquitin ligase mdm2 from its association with p53, thereby preventing mdm2-mediated nuclear export and subsequent proteasomal degradation of p53.
- The bLZip domain of Zhangfei is required for its profound effects on cell growth, the UPR and interaction with p53.

Chapter 5: Characteristics of the Interaction of p53 and Zhangfei

- p53 forms a complex with Zhangfei via its N-terminal transactivation domain (NTD), which is also the mdm2-binding domain and transcription activation region of p53.
- NTD is required for Zhangfei-mediated nuclear retention and transcriptional activation of p53.

Chapter 6: The Effect of Zhangfei/CREBZF on Cell Growth, Differentiation, Apoptosis, Migration, and the UPR in Several Canine Osteosarcoma Cell Lines

- Zhangfei suppresses the growth and UPR-related transcripts in several dog OS cell lines that express p53 proteins capable of activating promoters with p53 response elements, both on their own and synergistically with Zhangfei.
- Zhangfei induces the activation of osteocalcin expression, a marker of osteoblast differentiation and triggers programmed cell death in these dog OS cell lines.

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